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Award Number: W81XWH-06-1-0242

TITLE: Prostate Cancer Cell Growth: Stimulatory Role of Neurotensin And Mechanism of Inhibition by Flavonoids as Related to Protein Kinase C.

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REPORT DATE: January 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31-01-2008	Annual	1 Jan 2007 - 31 Dec 2007

4. TITLE AND SUBTITLE
Prostate Cancer Cell Growth: Stimulatory Role of Neurotensin and Mechanism of Inhibition by Flavonoids as Related to Protein Kinase C.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0242

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Massachusetts Medical School
Worcester, MA 10655

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose is to define the relationship between neurotensin (NT) and protein kinase C (PKC) isotypes and to investigate the mechanism by which flavonoids (FLAV) inhibit NT growth signaling in PC3 cells. The long-range scope is to determine the significance of NT in the negative effects of high fat intake on PC incidence and the positive effects of diets containing FLAV. Our results show that NT-induced growth signaling involves and requires activation of several PKC isotypes (most notably PKC epsilon and delta), that arachidonic acid metabolism and EGF receptor activation participate in the NT signaling process, that cell metabolism and ATP levels can influence NT receptor function, and that activated PKC (most notably PKC alpha and beta) can feed back to regulate the ability of NT receptor to bind NT and to initiate signaling. FLAV was found to exert differential effects on PKC isotype activation and downregulation. Thus, FLAV could alter the balance between conventional and novel PKC activity, which could influence growth responses to NT. These findings have implications regarding mechanisms that regulate NT receptor function and the design of agents to block NT-induced growth signaling in PC.

15. SUBJECT TERMS
neurotensin, PC3 cells, cancer growth, PKC isotypes, flavonoids

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	88	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Prostate Cancer cell growth: stimulatory role of neurotensin and the mechanism of inhibition by flavonoids as related to protein kinase C.

Principal Investigator, Robert E. Carraway

Second Year Report - Period 1/01/07 to 12/31/07

1. INTRODUCTION

Neurotensin (NT), an intestinal growth factor that is also involved in inflammation, has a potential role in prostate cancer (PC) since NT receptor (NTR) is over-expressed in PC and NT stimulates growth of cultured human PC cells. We found higher levels of NTR in aggressive androgen-independent PC3 cells than in the less aggressive androgen-dependent LNCaP cells. We demonstrated that NT stimulates DNA synthesis and growth of PC3 cells via a PKC dependent transactivation of EGF receptor. Fat intake, which correlates with PC incidence around the world, is the strongest stimulus for NT secretion, and NT promotes the conversion of arachidonic acid into growth-promoting eicosonoids (via lipoxygenase). Thus, NT and fatty acids could work in concert to mediate fat-induced PC growth. In contrast, a diet rich in antioxidant flavonoids (FLAV) correlates with decreased PC.

Our data shows that FLAV inhibit NT growth signaling in PC3 cells. Since FLAV can inhibit PKC and lipoxygenase *in vitro*, we are investigating the importance of PKC and lipoxygenase in the growth stimulating effects of NT. We hypothesize that NT released by dietary fat enhances PC growth and that FLAV inhibit NT growth signaling by inhibiting the activity of PKC and/or lipoxygenase. Since FLAVs inhibit NT signaling in cultured PC cells, this provides a rationale for the beneficial effects of Asian-Mediteranian diet. This year's work has focused on the role of PKC isotypes in NT growth signaling and on the effects of FLAV on PKC isotype expression, PKC isotype activity and NT growth signaling. Given the opposite growth roles of PKC-epsilon (stimulatory) and PKC-delta (inhibitory), it is important to identify the PKC-isotypes involved. Defining the mechanism by which FLAV inhibit NT-stimulated PC growth could shed light on general aspects of G-protein receptor action, signal transduction and pathway relationships, which could have a widespread impact due to the number of growth stimuli involved.

2. BODY

The work accomplished during this period is presented below according to the revised "Statement of Work" presented in the first year report submitted on January 31, 2007.

Task 1. Determine effect of FLAV on NT-induced PC3 tumor growth in mice (months 24-36).

This task was assigned to months 24-36; therefore, there is no work to report at this time.

Task 2. Determine effect of FLAV on LNCaP tumor growth in mice (months 34-36).

This task was assigned to months 24-36; therefore, there is no work to report at this time.

Task 3. Identify PKC isotypes that are activated by NT-induced growth signaling (months 1-15).

A. PKC isotype phosphorylation-

To initiate the work on task 3, we first examined the expression of PKC isotypes in the human prostate cancer cell lines PC3 and LNCaP cells. Western blotting was performed with specific antisera from Santa Cruz to detect various PKC isotypes in extracts of PC3 and LNCaP cells. Our results indicated that both cells lines expressed primarily the following PKC isotypes: α, β1, δ and ε; however, PC3 cells expressed relatively more PKCε and LNCaP cells expressed relatively more PKCδ (Fig 1 below). Since PKCε is generally growth enhancing and PKCδ is associated with apoptosis, these differences might account for the more aggressive nature of PC3 cells.

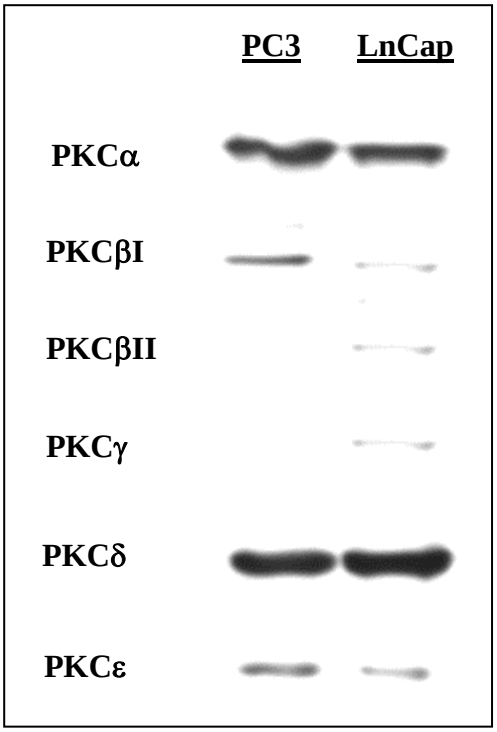


Fig 1. Expression of PKC isotypes in PC3 and LNCaP cells. An equal number of quiescent cells were processed for SDS-PAGE and western blotting using antisera specific for the PKC isotypes indicated.

Fig 2 (below) shows the results for PKC expression in control PC3 cells and for cells stimulated with PMA, which is known to downregulate PKC. Note that PMA downregulated the conventional PKCα and PKCβ1 much more effectively than the novel PKCδ and PKCε. This could be important in regards to growth regulation in these cells since PMA is growth stimulatory at low doses (which would not downregulate novel PKCs) and growth inhibitory at high doses (which would downregulate the novel PKCs). In contrast, NT activates PKCs without causing downregulation and thus, it is growth stimulatory at all doses. This led us to hypothesize that growth stimulation by NT and perhaps other inputs may be mediated more by novel PKCs than by conventional PKCs.

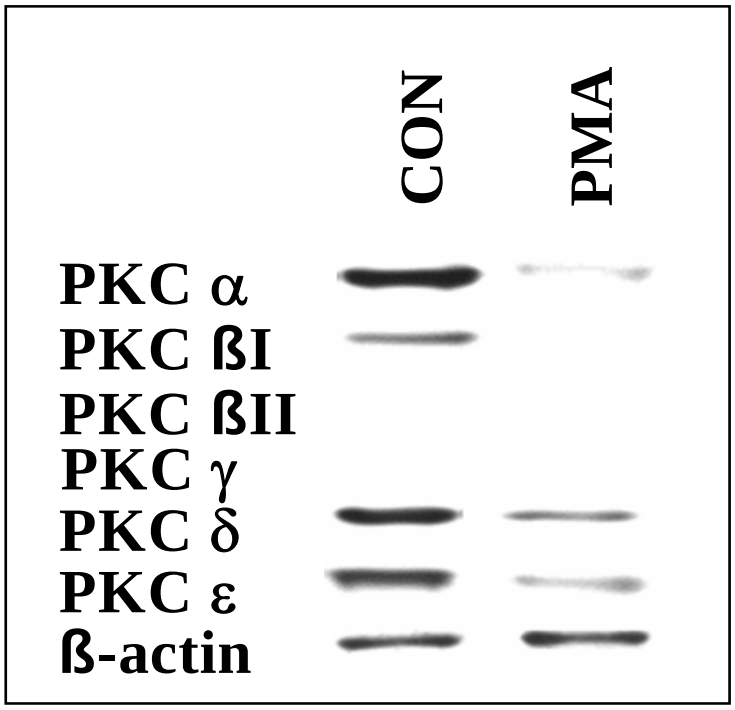


Fig 2. Wesern blot assessing expression of PKC isotypes in control PC3 cells and cells pretreated with PMA. Quiescent cells withdrawn from serum were treated for 24 hrs with 1 μM PMA or vehicle control and cell extracts (40 μg protein) were subjected to SDS-PAGE and western blotting using antisera specific for PKCα, PKCβ1, PKCβ2, PKCγ, PKCδ, and PKCε. β-actin was used as loading control.

Next, we set out to determine the PKC isotypes in PC3 cells that became phosphorylated in response to NT. Using antibodies to the phosphorylated forms of each PKC isotype, we determined the levels of PKC activation for control cells and NT treated cells. Western blotting was performed on cell extracts obtained after 1-30 min treatment. The cells were withdrawn from serum for 24 hrs before the medium was changed and NT was added. As reported last year, our initial studies indicated that the basal level of activation (constitutive activity) was so high that it was difficult to demonstrate an effect of NT. Experiments during the past year showed that our initial difficulties were due to mechanical effects on

the cells. We have solved this problem by adding NT from a 100 X solution to the quiescent cells, thus avoiding any mechanical disturbance (ie., without changing the medium). Under these conditions, exposure to NT for 5-30 min induced the phosphorylation of PKCα, PKCβ1, PKCδ and PKCε (Fig 3). In addition, we demonstrated that NT caused the phosphorylation of multiple conventional PKC substrates with molecular masses from 30 kDa to 140 kDa (Fig 3). Thus, in PC3 cells, NT activated the conventional PKCs (α, β1) as well as the novel PKCs (δ, ε).

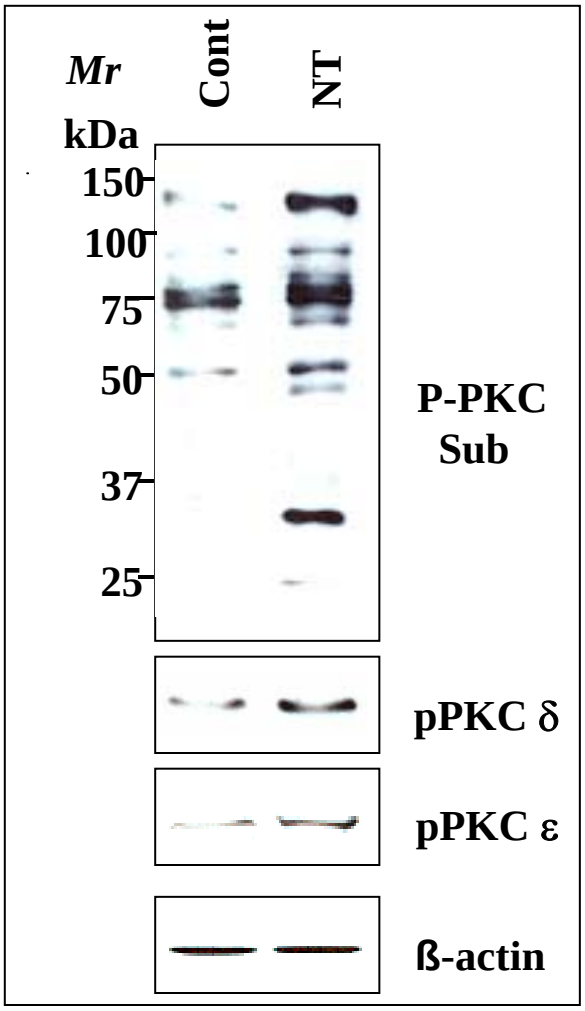


Fig 3. NT stimulates phosphorylation of multiple PKC substrates and activates (phosphorylates) both conventional and novel PKCs in PC3 cells. Quiescent cells, withdrawn from serum for 24 hrs, were stimulated with 10 nM NT for 30 min. Cell extracts were subjected to SDS-PAGE and western blotting. In the upper panel, endogenous PKC substrate phosphorylation was assessed using a phospho-specific antiserum to the motif R/K-X-S-Hyd-R/K. In the lower panels the activation of PKCδ and PKCε was assessed using antisera specific for phosphorylated forms of these PKC isotypes. β-actin was used as the loading control.

B. PKC isotype translocation-

To confirm these results, we then used the translocation assay which indicates PKC activation by it movement from cytosol to membrane. PC3 cells were treated with NT for 1-30 min as described above and after cell lysis, the membrane and cytosolic fractions were subjected to western blotting using antisera towards the individual PKC isotypes.

As explained in last year's report, our initial results indicated that the PKCs were often found in the membrane fraction under basal conditions, and thus, it was difficult to show an effect of NT. Again, our studies since then have shown that some of this difficulty was due to mechanical effects on the cells. Stimulating the cells by adding NT without changing the medium reduced the problem and allowed us to demonstrate that NT translocates PKC isotypes in PC3 cells. Translocation studies were performed using a conventional pan PKC antiserum, as well as antisera specific for PKCα, PKCδ and PKCε. As an example, Fig 4 shows results for PKCδ. Although it can be seen that NT stimulated the translocation of PKCδ and this was inhibited by quercetin and resveratrol, the effect was not striking and the results were not as convincing as those obtained with the phosphorylation assays. Therefore, we decided to primarily use the phosphorylation assays to assess PKC activation in response to NT and the various FLAV-like agents.

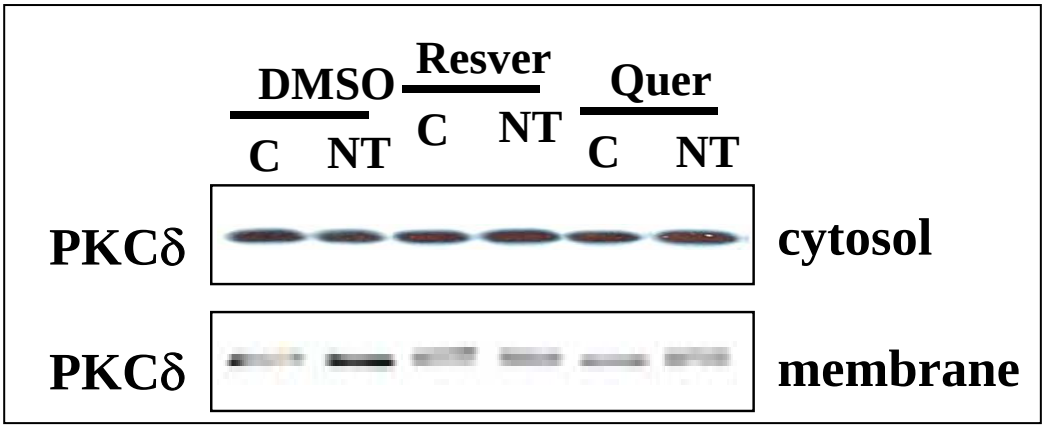


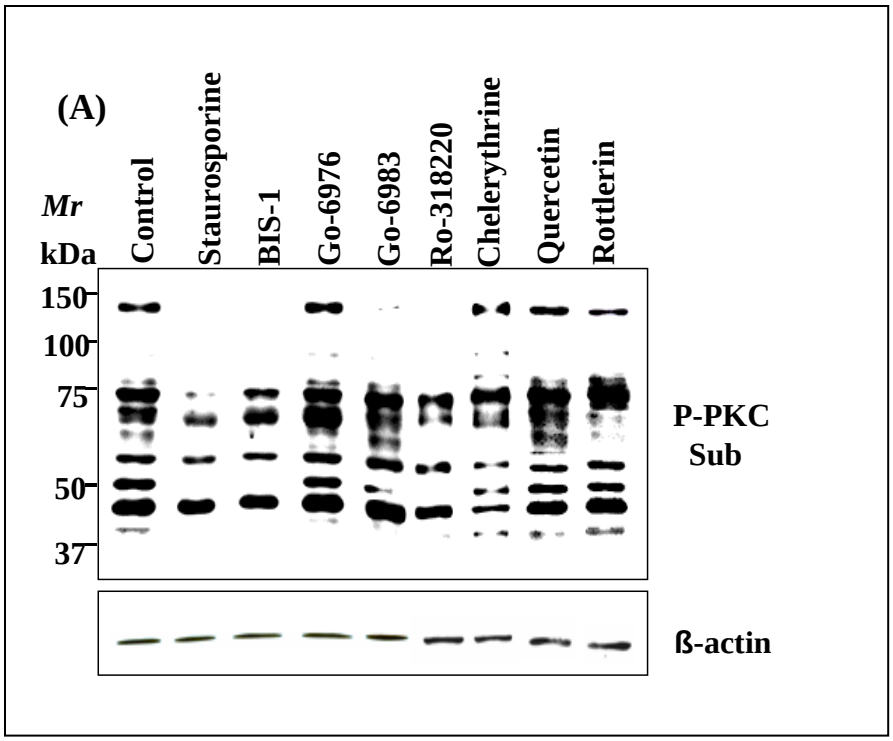
Fig 4. Quiescent cells were treated with 65 μM resveratrol or 35 μM quercetin or vehicle control for 30 min. Then, they were stimulated with 10 nM NT or control for an additional 20 min. Cell homogenates were centrifuged to isolate the cytosolic and membrane fractions, which were subjected to western blotting using an antiserum specific for PKCδ. Note that NT caused the translocation of PKCδ to the membrane fraction.

C. PKC isotype activity-

To assess cellular PKC activity, we choose to measure *in vivo* PKC substrate phosphorylation rather than to employ *ex-vivo* assays that use PKC immunoprecipitates to phosphorylate substrates in a test tube because only the former method could be certain to detect both direct and indirect effects of agents on PKC activity. Thus, the former method would but the later method would not detect any effects on PKC sequestration, substrate availability, cofactor and energy levels that would only exist *in vivo*.

Therefore, to examine the effects of NT and FLAV on cellular PKC activity, we used a western blotting assay to measure *in vivo* PKC substrate phosphorylation. The antiserum (Cell Signaling) was raised towards the phosphorylated PKC consensus motif R/K-X-S-Hyd-R/K. PKC isotype specificity considerations predicted that the assay would primarily detect substrates phosphorylated by conventional PKCs (α, β1, β2, γ) and was likely to respond to novel PKCε substrates and might possibly react (although poorly) with novel PKCδ substrates. Our results indicated that PC3 cells exhibited constitutive PKC activity and that NT caused a time-dependent (peak, 30 min) enhancement of PKC substrate phosphorylation (Fig 3 above).

The constitutive PKC substrate phosphorylation was inhibited differentially by a number of specific PKC inhibitors (Fig 5A and 5C), as was the PMA-induced PKC substrate phosphorylation (Fig 5B and 5D). Interestingly the inhibitory pattern for quercetin differed from those seen for other PKC inhibitors (best viewed in Fig 5C). Note that although the inhibitory effects of quercetin were not remarkable, quercetin appeared to specifically inhibit the phosphorylation of substrates with molecular masses of 60-85 kDa, without much effect on the 50 kDa band (Fig 5C). A similar pattern of inhibition was displayed by the specific PKCδ inhibitor rottlerin (Fig 5C), suggesting that quercetin specifically inhibited this novel PKC. In contrast, the specific inhibitor of conventional PKCs (Go-6976) had little effect on the 60-85 kDa substrates but instead very effectively inhibited the phosphorylation of the 50 kDa band (Fig 5C). These results supported the idea that quercetin was a specific inhibitor of novel PKCs (PKCδ and PKCε), and that this might explain the ability of quercetin to modulate NT receptor function.



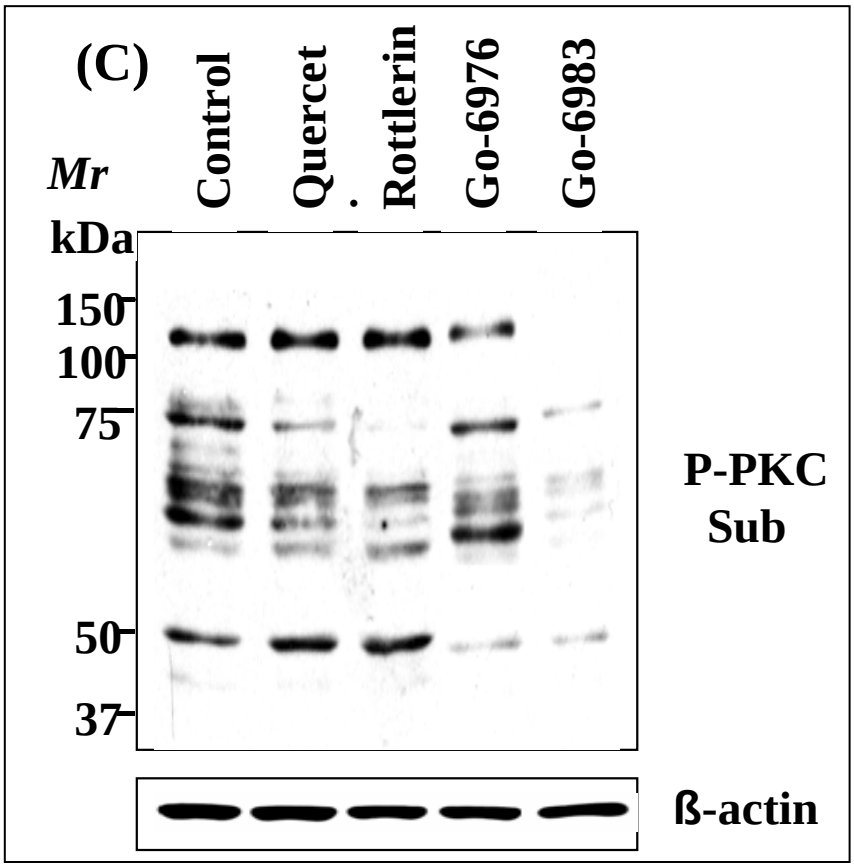
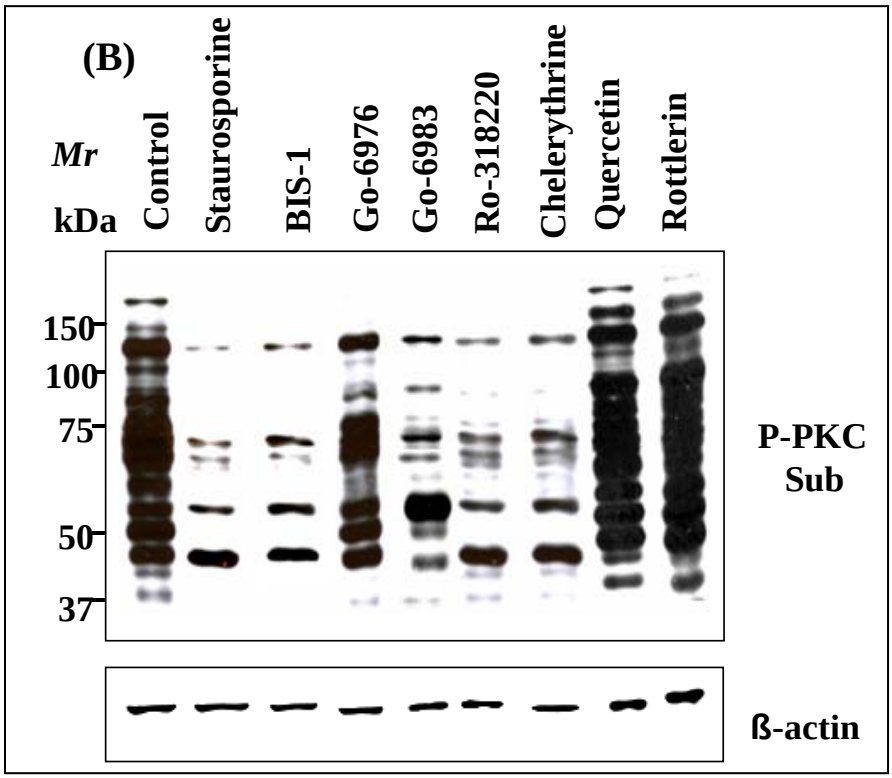


Fig 5A and 5C. Constitutive PKC activity was inhibited by BIS-1 and quercetin. Quiescent cells were incubated with inhibitors shown for 30 min and PKC substrate activity was determined by western blotting. In A, 15 μM quercetin was used. In C, 40 μM quercetin was used. Note that quercetin reduced the intensity of some of the bands (mostly those from 60-85 kDa). Contrasting the pattern for quercetin with that for Go-6976 suggested that quercetin inhibited novel PKCs rather than conventional PKCs.



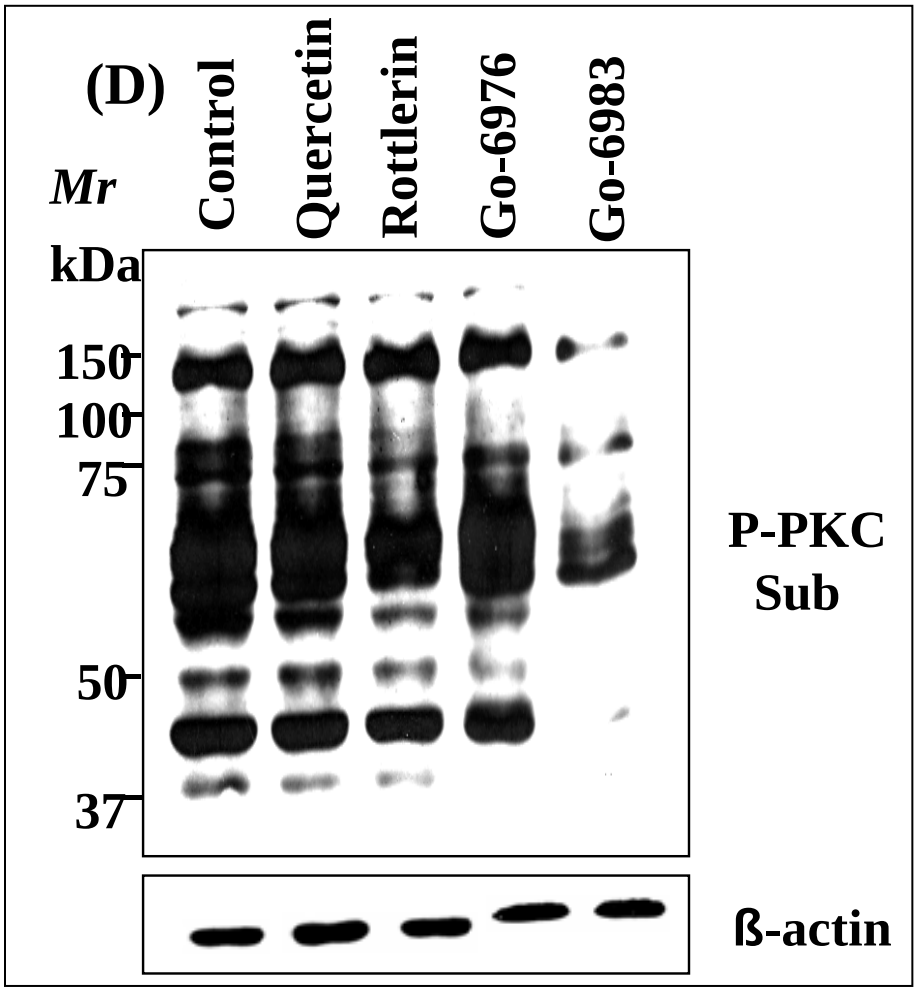


Fig 5B and 5D. PMA-induced PKC substrate activity was reduced by BIS-1 and quercetin. Cells were stimulated with 100 nM PMA for 30 min. In B, 15 μM quercetin and in C, 40 μM quercetin was used.

Task 4. Identify PKC isotypes that are required for NT-induced growth signaling (months 1-24).

A. Antisense Work-

PKC specific antisense oligodeoxynucleotide (ODN) sequences (20 mers) that were specific for PKCα, PKCδ and PKCε were obtained from Invitrogen. PC3 cells in Opti-MEM medium were exposed to lipofectamine (5μg/ml) mixed with the ODNs (1μM) for 3 days. Cell extracts were used to perform western blotting to quantitate changes in the expression of the individual PKC isotypes. The results indicated that PC3 cells were highly resistant to PKC knockdown by these methods (as reported last year). These negative results led us to investigate the usefulness of RNAi to perform these experiments.

B. RNAi work-

Plasmid DNAs containing shRNA sequences complementary to specific PKC isoforms were obtained from the UMass RNAi Core Facility and Open Biosystems. The sequences included: two silencing sequences for PKCα, two for PKCβ, two for PKCδ and one for PKCε, as well as a non-silencing control. The shRNAs were cloned in pSM2c vector under the control of the CMV promoter for expression in mammalian cells. A puromycin resistance gene was also present to facilitate the selection of clone pools.

Transient transfection was performed using FuGene (Roche) as recommended by the manufacturer. Basically, 5 x 10^4 cells/ml were plated per well of 12-well plates so that 40% confluence was obtained after 24 hrs. These were then transfected using various concentrations of FuGene (0.75-6 μl/ml) and of DNA (0.25-1.0 μg/ml). After 48 hrs, cell extracts were examined by western blotting to assess the effects on PKC expression. FuGene was toxic above 1.5 μl/ml. However, using this amount of FuGene with 0.5 μg DNA gave results consistent with specific knockdown (about 40%) for PKCδ and PKCε). We then performed experiments to test the effects of PKC knockdown on NT binding to the cells. Using our optimal conditions, cells were transfected with the non-silencing control and the silencing constructs, and NT binding was tested after 48 hrs. Basal binding was measured as well as binding in the presence of conventional and novel PKC inhibitors (1-2μM). Although some preliminary findings were obtained that were encouraging (as reported last year), the efficiency of the knockdown with transient transfection was not sufficient to produce reproducible and statistically significant results.

Therefore, we decided to select clone pools based on puromycin resistance. Several clones were obtained that displayed >50% knockdown of PKCδ and PKCε expression (Fig 6). Based on results from

western blotting, one clone from each treatment was selected for further analysis: nonsense (NS clone); PKCδ(-) clone; and PKCε(-) clone.

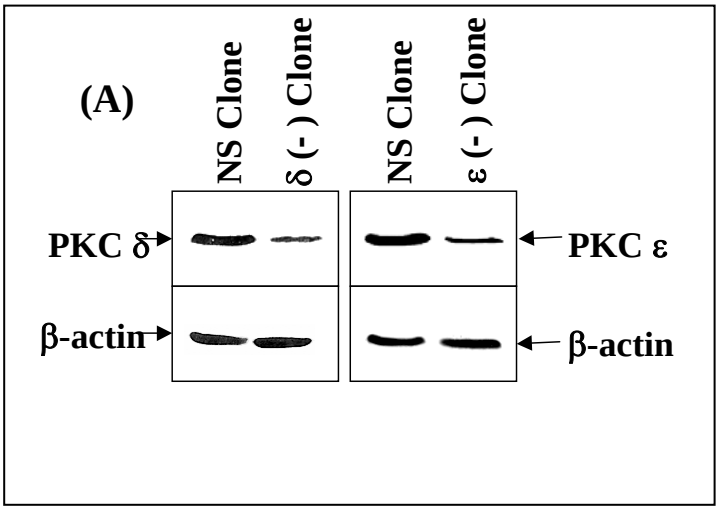


Fig 6. Expression of PKCδ and PKCε in the selected knockdown clones of PC3 cells. An equal number of cells was extracted and 40 ug of protein was subjected SDS-PAGE and western blotting with specific antisera was performed to assess PKC expression. β-actin was used as the loading control.

To determine the importance of PKCδ and PKCε in NT receptor function, we first measured basal NT binding in the clones when the cells were ≅90% confluent. Basal binding expressed as cpm/μg protein (mean±SEM, n=4) did not differ for the 3 clones (NS clone, 18.0±1.4; PKCδ(-) clone, 15.8±1.2; and PKCε(-) clone, 15.4±1.1). Then we assessed the effects of BIS-1 and rottlerin on cellular NT binding in these clones, hypothesizing that the enhancement of NT binding by these inhibitors might be reduced in clones that lacked the relevant PKC isotype. The results in Table 1 show that the responses in the different clones did not differ significantly. These results indicated that PKCδ knockdown or PKCε knockdown by itself was not sufficient to alter basal NT binding or the effects of BIS-1 and rottlerin on NT binding. One possible explanation was that knockdown of both PKCδ and PKCε (and perhaps PKCα and PKCβI) was necessary to reproduce the effects of these inhibitors on NT binding.

Table 1

Effect of PKC Inhibitors on NT Receptor Binding in PKC Knockdown and Control Clones of PC3 Cells

Cell Clone	NT Binding (% DMSO control)			
	BIS-1		Rottlerin	
	0.5μM	2.5μM	0.5μM	2.5μM
NS control	155±14	216±20	150±16	181±17
PKCδ (-)	168±13	254±17	145±13	222±18
NS control	140±13	225±19	140±14	236±19
PKCε (-)	167±12	268±21	177±15	244±18

NT receptor binding to each PC3 cell clone was measured in the presence of BIS-1 or rottlerin at the indicated concentrations as compared to the vehicle (DMSO) control. The cells (80-90% confluent) were preincubated 15 min with the agents in Locke prior to the binding reaction. Control NT binding for each clone was similar when expressed as cpm/μg protein: NS control, 18.0±1.4; PKCδ (-); 15.8±1.2; PKCε (-), 15.5±1.1 (n=4). For each clone, the effects of BIS-1 and rottlerin were expressed as % control NT binding (mean±SEM; n=3 experiments). The results for the different clones were not significantly different.

To further address the importance of PKC isotypes in NT receptor signaling, we next examined the ability of NT to stimulate inositol pohosphate (IP) formation in the various PKC knockdown clones. We found that NT was less effective in elevating IP formation in the PKCδ(-) and the PKCε(-) clones as compared to the NS clone (Fig 7). This was not due to a general effect on PLC activity or the level of phospholipid substrate since the IP response to 3nM BOM was not inhibited in the PKCδ(-) clone (% control response, 95±2; n=6) and was enhanced in the PKCε(-) clone (% control response, 148±13; n=6). These results suggested that PKCδ and PKCε were necessary for NT to maximally stimulate PLC to enhance inositol phosphate metabolism.

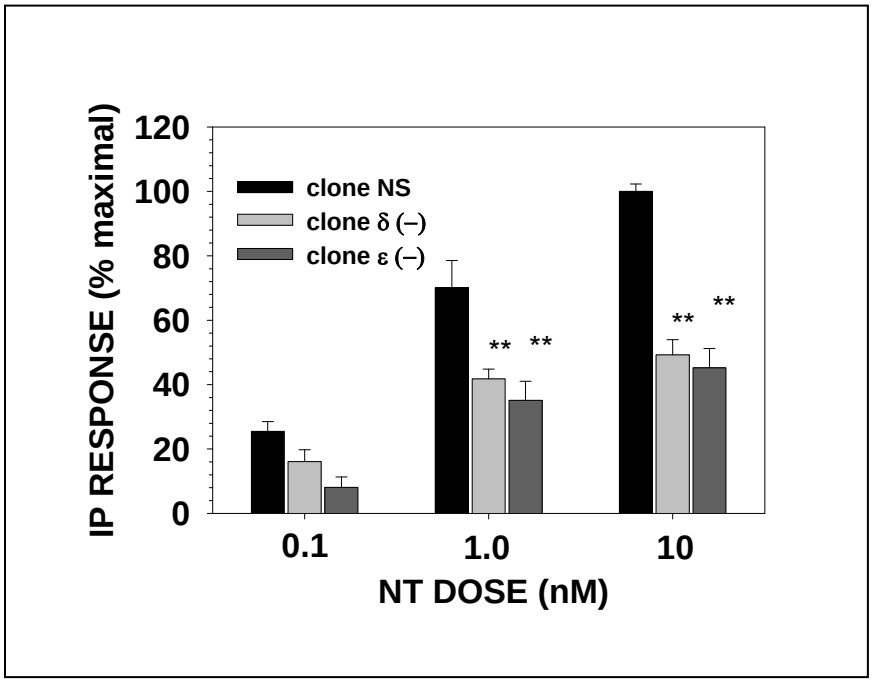


Fig 7. Effect of PKC knockdown on NT-induced IP formation. Equal numbers of cells were stimulated with the indicated doses of NT. IP formation was measured and calculated as % maximal.

These results show that knockdown of either PKCδ or PKCε was sufficient to diminish the IP response to NT. Consistent with the importance of both PKCδ and PKCε in this regulation, treatment with PKC inhibitor BIS-1 further inhibited the NT response and further enhanced the BOM response, even in the PKCδ(-) and PKCε(-) clones (Fig 8). These results were in keeping with the hypothesis that both PKCδ and PKCε activity were required to maintain the ability of NT receptor to stimulate PLC.

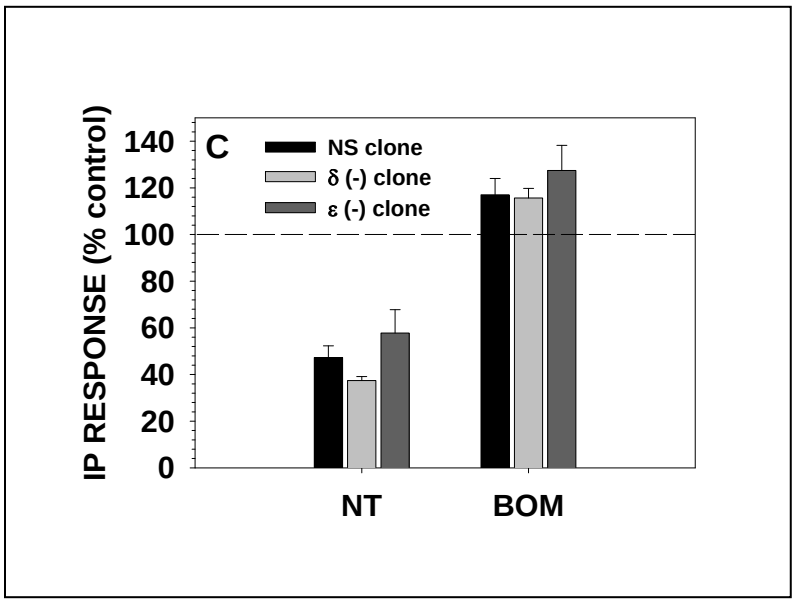


Fig 8. BIS-1 inhibited the effect of NT and enhanced the effect of BOM on IP formation even in the PKCδ(-) and PKCε(-) clones. An equal number of cells, pretreated with 2.5 μM BIS-1 or vehicle control, were stimulated with 10 nM NT or 3 nM BOM. The IP responses were calculated as % control for each individual clone. In the NS clone, IP formation was elevated about 4-fold by NT and about 10-fold by BOM. In the PKCδ(-) and PKCε(-) clones, the control response to NT was about 50% less than in the NS clone, but this response was further inhibited by BIS-1, showing that both PKCδ and PKCε participated in this regulation.

Task 5. Determine effects of FLAV on activation and expression of PKC isotypes (months 12-24).

A- Effects of FLAV on PKC activation by NT.

Next we assessed the effect of FLAV on NT-induced *in vivo* PKC substrate phosphorylation using the western blotting method previously described. NT enhanced PKC substrate phosphorylation, increasing the intensity of bands in the size range of 40-140 kDa (Fig 9). The effects of NT were inhibited by quercetin and BIS-1 (Fig 9). Note that the effects of quercetin again appeared to be selective for PKC substrates in the size range of 60-85 kDa. This led us to hypothesize that these substrates were primarily PKCδ and PKCε substrates. We also showed that the response to NT involved the activation (phosphorylation) of PKCα, PKCβ1, PKCδ and PKCε. Fig 9 shows the activation of PKCδ and PKCε by NT, and also the fact that quercetin and BIS-1 inhibited both the basal and NT-induced phosphorylation of PKCδ and PKCε.

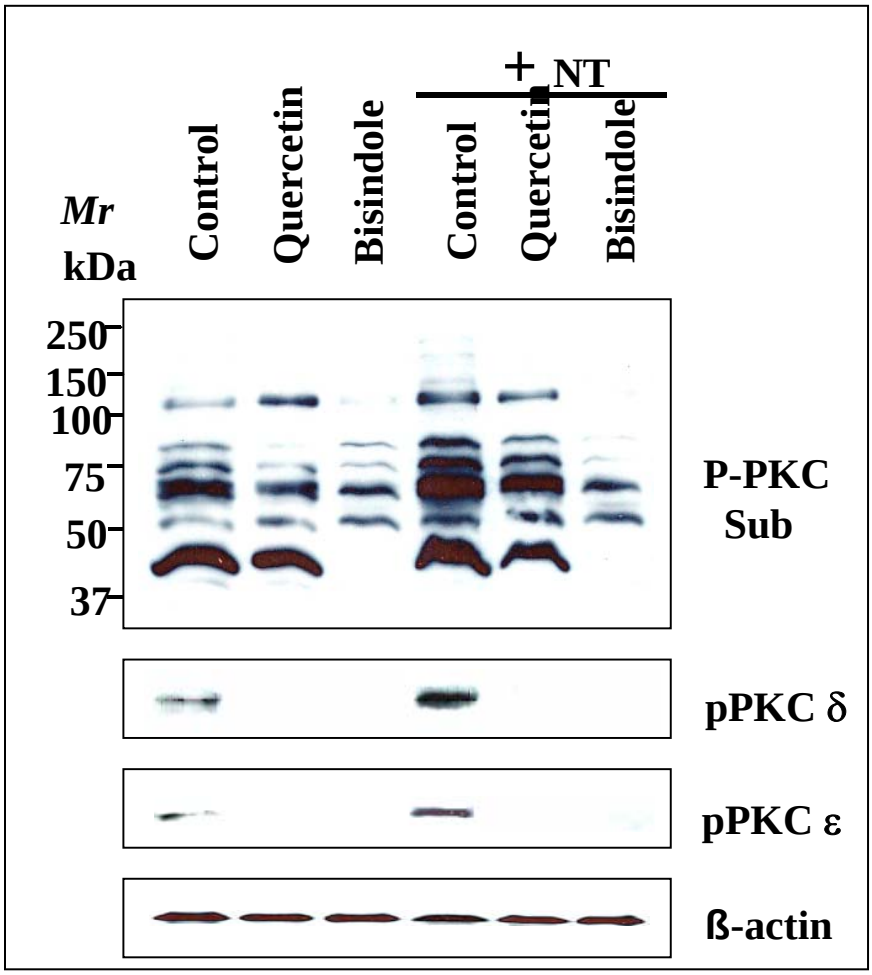


Fig 9. Effect of quercetin and BIS-1 on control and NT-induced PKC substrate phosphorylation and on the activity level (phosphorylation) of PKCδ and PKCε. Quiescent cells were pretreated with 35μM quercetin or 7 μM BIS-1 or vehicle control, and then stimulated with 30 nM NT for 30 min. Cell extracts were processed for western blotting using phospho-specific antisera towards PKC substrates and PKC isotypes. β-actin was used as loading control.

B- Effects of FLAV on PKC Expression in PC3 cells.

Quercetin and BIS-1 induced a dramatic downregulation of novel PKCε and PKCδ, whereas the conventional PKCα was upregulated (Fig 10). Again, this supported our hypothesis that quercetin exerted specific effects on the novel PKCδ and PKCε.

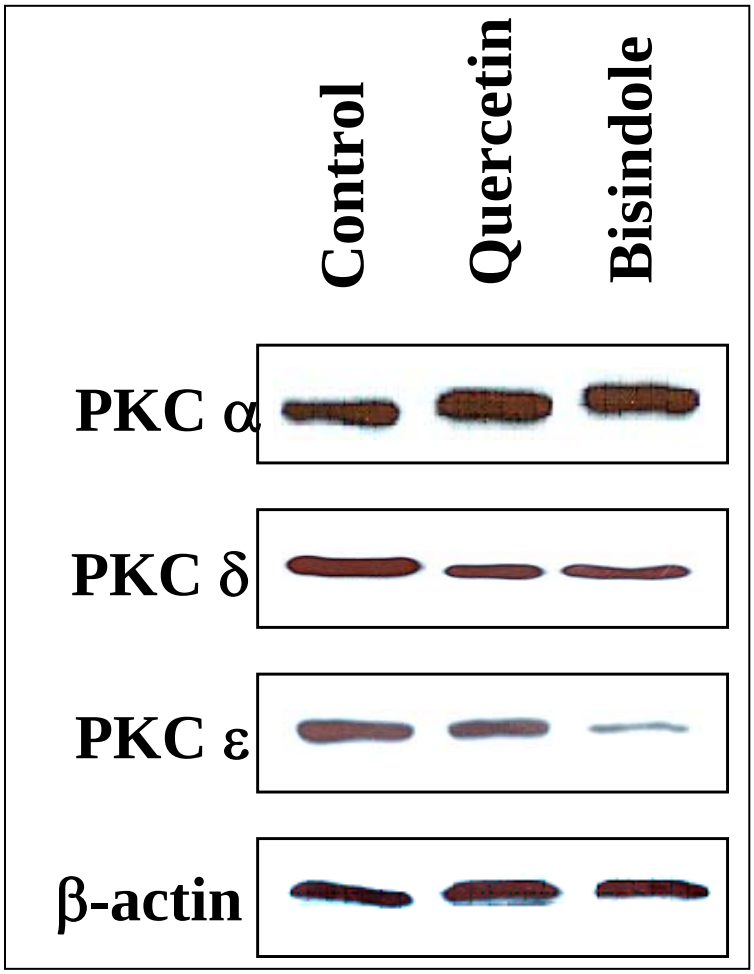


Fig 10. Quiescent cells were incubated with 35 μM quercetin or 7μM BIS-1 or vehicle control for 20 hrs and then cell extracts were subjected to SDS-PAGE and western blotting using antisera specific for PKCα, PKCδ and PKCε. β-actin was used as the loading control.

The time-course of the effect of quercetin and BIS-1 on PKCε expression is shown in Fig 11. Both of these agents downregulated PKCε time-dependently and this downregulation was noticeable after 30 min and very significant after 4 hrs exposure to 7 μM BIS-1 or 100 μM quercetin (high dose), while the low dose of quercetin (35 μM) took a longer time to develop. We hypothesize that the inhibition and downregulation of PKCδ and PKCε by quercetin and BIS-1 relate to their effects on NT receptor function. Although these agents alter NT receptor function within 15-30 min, which is well before downregulation was seen, it could be that these PKCs are sequestered as they enter the downregulation pathway and that this is sufficient to inhibit NT receptor function. In other words, the rapid effects of quercetin and BIS-1 on NT receptor function could be an early reflection of the sequestration of PKCδ and PKCε (perhaps within vesicles) as they move along the degradative pathway towards lysosomes.

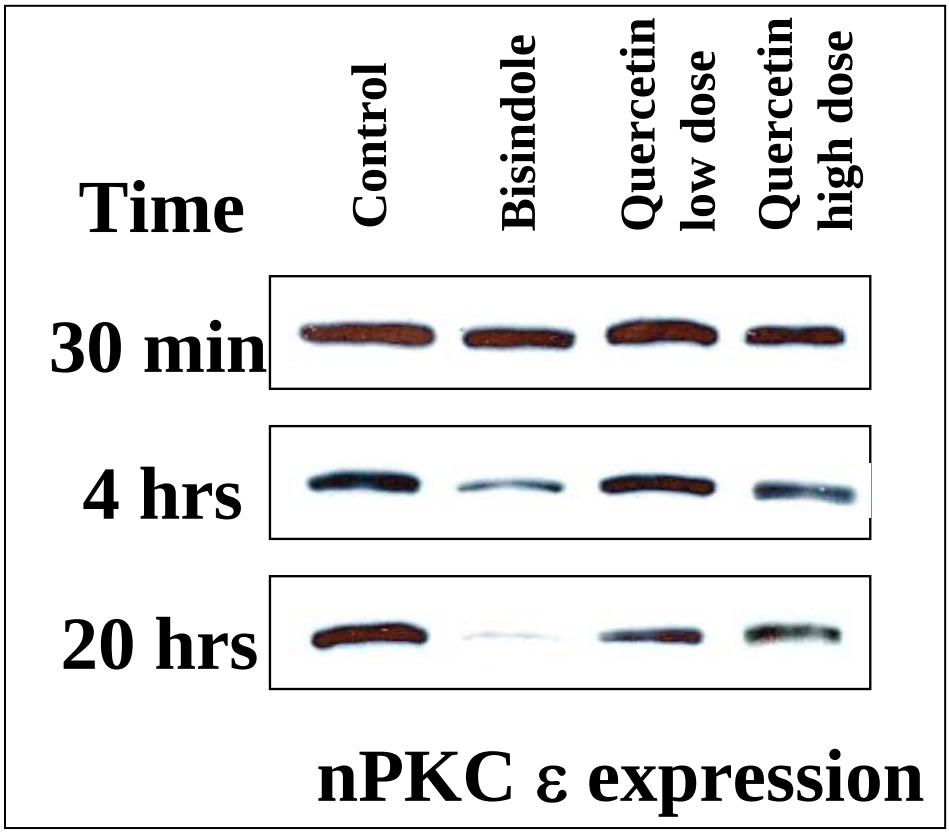


Fig 11. Time course for effects of quercetin and BIS-1 on PKCε expression. Quiescent PC3 cells were incubated with 7 μM BIS-1 or 35 μM quercetin (low dose) or 100 μM quercetin (high dose) or vehicle control for the times indicated. Cell extracts were subjected to western blotting using an antiserum specific for nPKCε. β-actin was used as the loading control.

C- Effect of FLAV on cellular ATP levels.

It is conceivable that inhibition of PKC enzyme activity and/or inhibition of its movement to membranes might involve changes in cellular ATP levels. In our manuscript provided in last year's report (Carraway et al, Regulatory Peptides 141, 140-153, 2007) we showed that NT receptor binding and signaling were sensitive to metabolic inhibitors. Glycolytic and mitochondrial inhibitors, which reduced cellular ATP levels and activated AMP kinase, produced the same effects on NT growth signaling that are seen in the presence of FLAV. Therefore, we tested the effects of quercetin and BIS-1 on cellular ATP levels. The results in Fig 12 indicate that quercetin and BIS-1, tested across the concentration range that altered NT receptor signaling, had no significant effect on cellular ATP levels. In contrast, antimycin A (which served as the positive control) dramatically reduced cellular ATP levels across the concentration range that altered NT receptor binding and signaling (Fig 12). Although it is still unclear whether metabolic inhibitors affect NT signaling via effects on novel PKCs, these results indicate that quercetin and BIS-1 do not dramatically alter cellular metabolism.

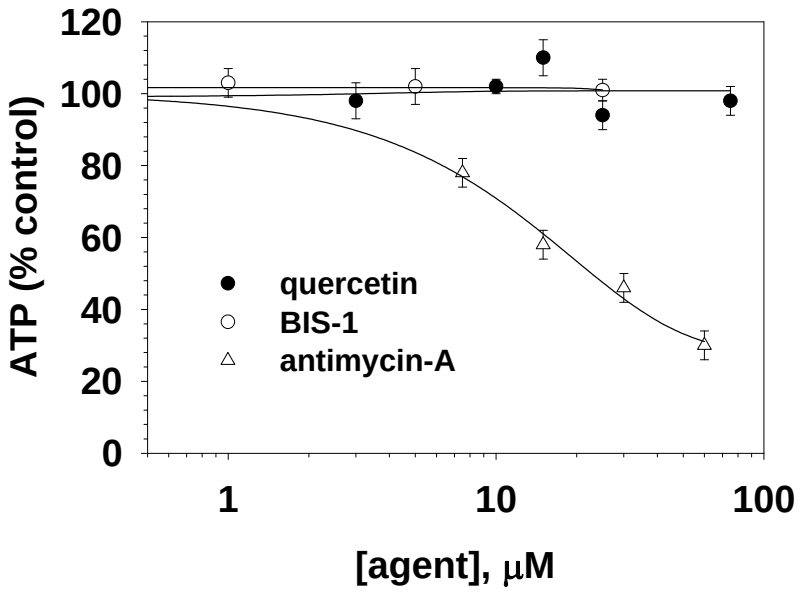


Fig 12. Quercetin and BIS-1 do not affect PKC activity via effects on cellular metabolism. Quiescent cells were incubated with the indicated concentrations of quercetin, BIS-1 or antimycin A for 45 min. Cells were extracted with TCA and cellular levels of ATP were measured using a luciferase based assay.

Task 6. Determine effects of FLAV on NT signaling modules in caveolae (months 20-30).

These studies are still in progress. Using ultracentrifugation in a sucrose gradient, we have demonstrated the localization of NT receptor in light density membranes that contain caveolin. Disruption of caveolae using β-cyclodextrin (which adsorbs cholesterol) caused caveolin, NT receptor, Gαq and PLCβ to move from the light membranes to the heavy membrane fraction (Fig 13). Thus, the NT signaling modules appeared to be disrupted by β-cyclodextrin. Interestingly, quercetin and BIS-1 also shifted these proteins and caused degradation of NT receptor. Consistent with the similar effects seen here on the distribution of these proteins, β-cyclodextrin also reproduced the effects of quercetin and BIS-1 on NT receptor binding and signaling. These results suggested that NT signaling modules can be disrupted by removal of cholesterol from the cellular membranes or by agents such as quercetin and BIS-1 which appear to inhibit novel PKC activity. It is still unclear whether β-cyclodextrin inhibits novel PKC activity and also whether quercetin and BIS-1 displace cholesterol from cellular membranes.

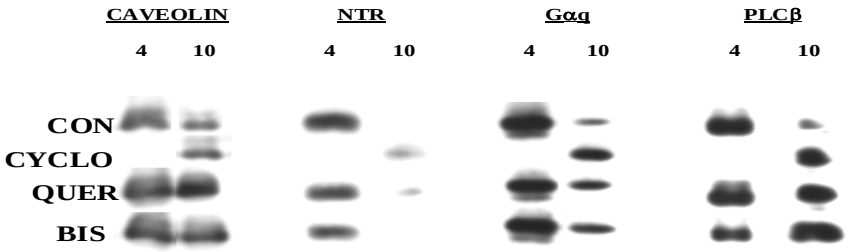


Fig 13. Disruption of NT signaling modules by cholesterol absorption (using β-cyclodextrin) and by inhibition of novel PKCs (using quercetin and BIS-1). Quiescent cells were treated with 5 mM β-cyclodextrin or 50 μM quercetin or 10 μM BIS-1. Sucrose centrifugation was used to separate caveolae from heavy membranes. Western blotting was performed for caveolin, NT receptor, Gαq and PLCβ in the 10 fractions obtained. Shown are the results for the peak fractions (#4 = caveolae; #10 = dense membranes). For the control, all of the NT receptor and most of caveolin, Gαq and PLCβ were in fraction #4. β-cyclodextrin caused caveolin, NT receptor, Gαq and PLCβ to move from fraction #4 to #10. Quercetin and BIS-1 also shifted these proteins and caused degradation of NT receptor.

Do the effects of FLAV on NT receptor correlate to their effects on cancer cell growth?

It is well established that FLAV exert inhibitory effects on cancer cell growth while having much less effect on the growth of normal cells. Although the long-term effects (>24 hrs) of these agents in cancer cells have been described, almost nothing is known about the rapid effects that initiate them. For example, quercetin can stimulate the classical mitochondrial apoptotic pathway and also enhance cell killing in response to death receptor activation. However, the mechanisms are not well understood because the changes that have been studied usually begin after 8-12 hrs or later. NT provides a system where the rapid effects (5-30 min) of FLAV can be studied using assays that yield results within hours. If the effects of FLAV on the NT system correlate to those on cancer cell growth, NT assays might be used as screening tools to facilitate the identification of more potent agents in the FLAV category that might be appropriate for therapeutic uses.

The results in Fig 14 relate the effects of various FLAV compounds on NT receptor binding to those on the growth of cultured PC3 cells. Note that the EC50 to alter NT binding correlates to the IC50 to inhibit cell growth. This suggests that FLAV may target the same mediators in producing these effects. Our data at this point suggest that the inhibiton or sequestration of novel PKCs could be an important early step.

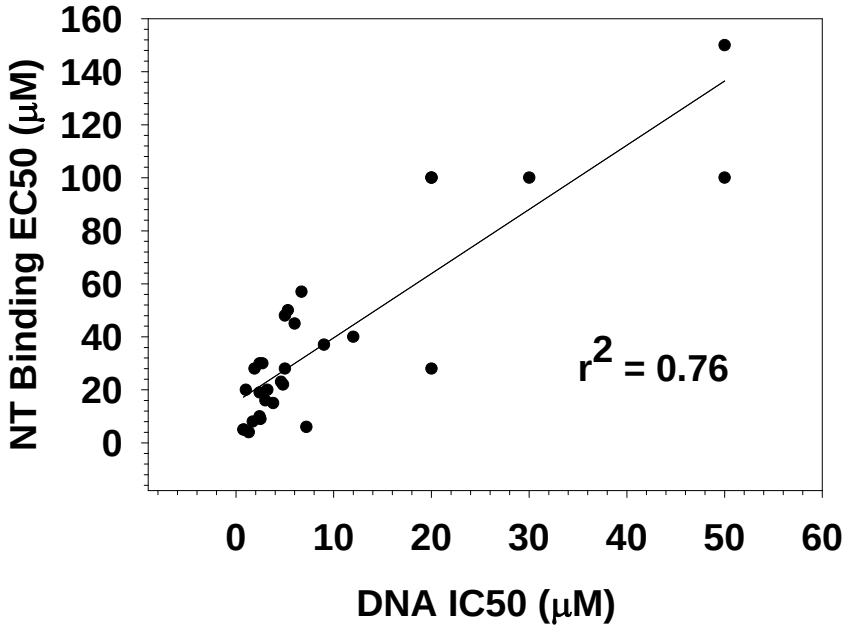


Fig 14. The potency for FLAV to alter NT receptor binding correlates to the potency to inhibit PC3 cell growth. Dose-response assays were performed to measure the effects of FLAV compounds on NT receptor binding to intact cells (during 30 min) and the EC50 was determined for each agent. Similarly, dose-response studies were performed using a 2-day assay for cell growth that measured ^{3}H-thymidine incorporation into DNA, and the IC50 was determined for each agent.

3. KEY RESEARCH ACCOMPLISHMENTS-

- Demonstrated that lipoxygenase inhibitors mimicked FLAV effects on NT receptor.
- Demonstrated that metabolic inhibitors reduced ATP and mimicked FLAV effects on NT receptor.
- Demonstrated that sub-class of PKC inhibitors (BIS-1 like) mimicked FLAV effects on NT receptor.
- Demonstrated that FLAV inhibited and downregulated novel PKCδ and PKCε.
- Demonstrated that knockdown of either PKCδ and PKCε mimicked FLAV effects on NT receptor.
- Demonstrated that disruption of caveolae (by removing cholesterol from membranes) mimicked FLAV effects on NT receptor.
- Demonstrated that FLAV were not metabolic inhibitors (did not reduce cellular ATP).
- Concluded that novel PKCδ and PKCε maintain NT receptor signaling modules in the working state, and that this maintenance process requires ATP, cholesterol and lipoxygenase activity.

4. REPORTABLE OUTCOMES-

A. Regulatory Peptides, 133:105-114, 2006

We knew from the outset of this grant work that fatty acids, including arachidonic acid (AA) and the AA metabolites (HETES) resulting from the action of lipoxygenases, could increase PKC activity in cell preparations. Therefore, for the purposes of this grant, it was important to define the relationship between NT and the AA cascade in regards to the regulation of PKC and NT-induced growth signaling. Thus, to compliment the studies described here, we examined the effects of AA pathway inhibitors on NT-induced growth signaling in PC3 cells. Our findings support the idea that the growth effects of NT involve a feed forward system whereby NT releases AA metabolites which enhance its effects on PKC, and that PKC mediates the activation of the EGF / MAP kinase cascade. The manuscript describing this work (Involvement of arachidonic acid metabolism and EGF receptor in neurotensin-induced prostate cancer PC3 cell growth. Regulatory Peptides, 133:105-114, 2006) was attached to the file for the first year's report.

B. Prostaglandins, Leukotrienes and Essential Fatty Acids, 74:93-107, 2006

Since the AA cascade interacts with PKC, and PKC inhibitors alter NT binding (see above), it was also important for the purposes of this grant to define the effects of the AA cascade on NT binding in PC3 cells. During this period, we performed some studies in this area, and our results support the idea that the NT receptor is regulated by specific feedback mechanisms involving lipid peroxidation and/or lipoxygenase-dependent reactions. The manuscript describing this work (Regulation of neurotensin receptor function by the arachidonic acid lipoxygenase pathway in prostate cancer PC3 cells. Prost Leuk Essen Fatty Acids 74:93-107, 2006) was attached to the file for the first year's report.

C. Regulatory Peptides, 141, 140-153, 2007

We also knew from the outset of this project that cellular metabolism and the formation of ATP was likely to influence PKC activity since all kinases require ATP. Therefore, for the purposes of this grant it was important to define the effects of general metabolism on NT receptor binding and NT-induced growth signaling. We performed some studies during this period, and our work indicated that metabolic inhibitors displayed effects on NT binding and signaling that were similar to those seen with PKC inhibitors. These findings are germane to the current studies because they further define the mechanisms regulating the interactions of NT and PKC. In addition, they underline the importance of defining the general metabolic effects of the agents under study. Overall, this work indicated that NT receptor function was sensitive to metabolism. Other preliminary data suggests that some of this regulation could

be exerted by way of PKC since metabolic inhibitors diminished PKC substrate phosphoryation in PC3 cells. The manuscript describing this study (Neurotensin receptor binding and neurotensin-induced growth signaling in prostate cancer PC3 cells are sensitive to metabolic stress. Regul Peptides 141, 140-153, 2007) was attached to the file for the first year report.

D. Regulatory Peptides in press, 2008

This reports the effects of quercetin and other PKC inhibitors on NT receptor binding and growth signaling. The findings suggested that PKC participates in heterologous regulation of NT receptor function by two mechanisms: a0- conventional PKCs inhibit NT receptor binding and signaling; b)- novel PKCs maintain the ability of NT to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NT receptor is also subject to homologous regulation by PKC. This manuscript (Protein kinase C inhibitors alter neurotensin receptor binding and function in prostate cancer PC3 cells) is attached to this file.

E. Peptides 27:2445-2460, 2006

For the purposes of this grant, it is also important to review the literature. In this review, we evaluated the evidence that NT and NT receptors participate in cancer growth and we described possible mechanisms. In addition, we described the progress achieved in the use of NT analogs to image tumors in animals and humans. This manuscript (Involvement of neurotensin in cancer growth: evidence, mechanisms and development of diagnostic tools. Peptides, 27:2445-2460) was attached to the file for the first year's report.

5. CONCLUSIONS-

Our results show that the NT receptor is subject to homologous regulation by PKC and that there appear to be two mechanisms. One mechanism involves negative effects on NT receptor binding and signaling which seem to be brought about primarily by conventional PKCs (α, β). A second mechanism has to do with positive effects on the maintenance of NT receptor signaling modules which appear to involve the actions of novel PKCs (δ, ε). Polyphenols and flavonoids, which are known to inhibit PC growth, can exert multiple effects on NT receptor function leading to an inhibition of growth signaling. Our studies on quercetin indicate that it appears to specifically inhibit and downregulate novel PKCs, and this may be the mechanism by which it disrupts NT receptor signaling.

The relevance of this work to the regulation of PC growth and to the development of useful therapeutics is shown by the fact that the effects of various flavonoids on NT receptor binding correlate to those on DNA synthesis in cultured PC cells. Thus, the mechanism by which flavonoids alter NT receptor function may be similar to that by which flavonoids inhibit the growth of PC cells. Our studies point towards a mechanism involving the inhibition and downregulation of novel PKCs, resulting in an upset in the balance between conventional PKC activity and novel PKC activity. Although this is not absolutely proven yet, it seems that an over-abundance of conventional PKC activity desensitizes NT receptor (and perhaps other growth receptors as well), leading to growth inhibition. On the other hand, an over-abundance of novel PKC activity maintains NT signaling modules (and perhaps modules for other growth receptors), leading to growth enhancement.

This model appears to explain a number of things in the literature. One enigma is that NT, BOM and ATP all activate PKC and lead to growth stimulation, whereas PMA activates PKC and leads to growth inhibition. This may be explained by the downregulation of novel PKCs that occurs only in response to PMA. Although PMA also downregulates the conventional PKCs, this may not be important since BIS-1 and quercetin, which downregulate only the novel PKCs, lead to growth inhibition.

6. REFERENCES-

The references are listed and discussed in REPORTABLE OUTCOMES. In addition these manuscripts are in the APPENDIX of the first year's report and of this report.

7. APPENDIX-

Elsevier Editorial System(tm) for Regulatory Peptides
Manuscript Draft

Manuscript Number: REGPEP-D-07-00039R1

Title: Protein Kinase C Inhibitors Alter Neurotensin Receptor Binding and Function in Prostate Cancer PC3 Cells

Article Type: Full Length Article

Keywords: Neurotensin; Prostate Cancer; Protein Kinase C; G Protein Binding; G Protein Signaling.

Corresponding Author: Mr robert Edward carraway, Ph.D.

Corresponding Author's Institution: University of Massachusetts Medical School

First Author: robert Edward carraway, Ph.D.

Order of Authors: robert Edward carraway, Ph.D.; Sazzad Hassan, Ph.D.; Paul R Dobner, Ph.D.

Abstract: Abstract

Prostate cancer PC3 cells expressed constitutive protein kinase C (PKC) activity that under basal conditions suppressed neurotensin (NT) receptor function. The endogenous PKC activity, assessed using a cell-based PKC substrate phosphorylation assay, was diminished by PKC inhibitors and enhanced by phorbol myristic acid (PMA). Accordingly, PKC inhibitors (staurosporine, Go-6976, Go-6983, Ro-318220, BIS-1, chelerythrine, rottlerin, quercetin) enhanced NT receptor binding and NT-induced inositol phosphate (IP) formation. In contrast, PMA inhibited these functions. The cells expressed conventional PKCs (☐, ☐ I) and novel PKCs (☐, ☐), and the effects of PKC inhibitors on NT binding were blocked by PKC downregulation. The inhibition of NT binding by PMA was enhanced by okadaic acid and blocked by PKC inhibitors. However, when some PKC inhibitors (rottlerin, BIS-1, Ro-318220, Go-69830, quercetin) were used at higher concentrations (>2 ☐ M), they had a different effect characterized by a dramatic increase in NT binding and an inhibition of NT-induced IP formation. The specificity of the agents implicated novel PKCs in this response and indeed, the inhibition of NT-induced IP formation was reproduced by PKC ☐ or PKC ☐ knockdown. The inhibition of IP formation appeared to be specific to NT since it was not observed in response to bombesin. Scatchard analyses indicated that the PKC-directed agents modulated NT receptor

affinity, not receptor number or receptor internalization. These findings suggest that PKC participates in heterologous regulation of NT receptor function by two mechanisms: a)- conventional PKCs inhibit NT receptor binding and signaling; b)- novel PKCs maintain the ability of NT to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NT receptor is also subject to homologous regulation by PKC.

Dr. Juris J. Meier, M.D., Associate Editor
Dr. Wolfgang E. Schmidt, M.D., Ph.D., Editor in Chief
Regulatory Peptides November 30, 2007

Dear Editors and Manuscript Reviewers: RE: REGPEP-D-07-00039

With this letter, we are submitting a revision of the above referenced manuscript, which upon initial review was considered suitable for publication in Regulatory Peptides contingent on revision according to the reviewers' comments. We have addressed all of the issues raised by the reviewers and complied with many of the reviewers' suggestions. Below we list each of the points made by the reviewer, and then summarize and discuss each of our responses.

Reviewer Comment #1- In short the implication is that neurotensin can activate PKC and dependent upon the predominant PKC family member, this can serve to inhibit or potentiate further the response to neurotensin.

Response - Although we did mention in the final sentence of the original Abstract that "homologous" regulation of NT receptor was possible, our main point was that the results of the current study indicate "heterologous" regulation of NT receptor by constitutively active PKC. It may be important to distinguish these two since most of the current study focuses on PKC activity that is evident under basal conditions and in response to activator PMA but not in response to NT stimulation. However the reviewer raises a very important issue here which is difficult to resolve. The traditional view of receptor binding is that the very low concentration of ^{125}I-labeled NT ligand used does not activate or desensitize a significant number of receptors, and therefore, the measurements of affinity and receptor number reflect the unstimulated state. However, it is conceivable (in my mind) that the "basal" binding parameters actually reflect a desensitized state that comes about by the local activation of the receptors that are hit with the labeled ligand and the local feedback effect of activated PKC. In other words, the binding enhancement by PKC inhibitor could reflect an inhibition of constitutively active PKC or an inhibition of the feedback process. At the present time, we have chosen the former interpretation since the PKC substrate phosphorylation assay shows that the cells display "basal" activity in the absence of NT or other PKC activators, presumably from constitutively active PKC. We also do not discuss this point since we have no evidence to challenge the current dogma regarding the interpretation of binding data.

Reviewer Comment #2 - I would like to see an attempt to knockdown PKC delta using an RNAi approach to test their proposed mechanism more directly. This can in principle be extended to the other PKC family members.

Response - Using puromycin to facilitate clone selection, we have now successfully obtained several clones exhibiting >50% knockdown of one of the novel PKC subtypes (PKC delta or PKC epsilon) as well as clones from scrambled RNAi controls. We have examined the behavior of these clones in the NT binding assay (results are presented in 3.6 of the revised manuscript) and in the IP assay (results presented in 3.7 of revised manuscript). Our studies show that knockdown of either PKC delta or PKC epsilon did not alter basal NT binding and did not change the response to category II PKC inhibitors (Table 1, revised manuscript). However, these knockdown clones displayed a decreased ability to activate PLC in response to NT (Fig 6, revised). These results indicated that knockdown of PKC delta or PKC epsilon by itself was not sufficient to negate the effects of these PKC inhibitors on NT binding, supporting the idea that several PKCs (most likely conventional PKC alpha and PKC beta) regulate NT binding. On the other hand, the IP results support a role for PKC delta and PKC epsilon in regulating receptor-effector coupling. These results are presented, interpreted and extensively discussed in the sections of the revised manuscript that are highlighted. In short, we are very pleased that the reviewer suggest these experiments since the results support our current model, which is that conventional PKCs regulate NT binding affinity whereas novel PKCs regulate receptor-effector coupling. The differential

effects of the various PKC inhibitors can be largely explained on the basis of their ability to dose-dependently and disproportionately inhibit these PKC isotypes.

Reviewer Comment #3- In order to comprehensively rule out the possibility that these effect are mediated through alterations in the trafficking/internalization of receptors I would like to see the assays repeated in combination with a panel of dominant negative constructs targeting endocytic trafficking.

Response- We have addressed this point and our results presented in section 3.9 and Fig 8 (revised manuscript) indicate that inhibition of clathrin-coated vesicle-mediated endocytosis using sucrose did not alter the effects of BIS-1on NT cell-surface binding or on NT internalization. BIS-1 worked despite blocking clathrin-coated vesicle endocytosis. The fact that sucrose inhibited clathrin-coated internalization in PC3 cells was shown using the bombesin receptor as a positive control (known to use this pathway). These results support our other results (Fig 7) showing that BIS-1 and PMA alter both cell-surface and internal binding, and (Fig 9) showing that BIS-1 and PMA modulate NT receptor affinity. None of these agents alter the internalization expressed as percentage of total binding. Thus, the evidence is overwhelming that PKC inhibitors enhance NT binding by an effect on receptor affinity that translates into more binding to the cell surface and as a result more internalization, but the internalization process itself does not appear to be modulated by BIS-1. Also, bombesin binding was not much affected by BIS-1 and bombesin internalization was not enhanced by BIS-1.

Comments to Editor and reviewers- We are very pleased with the quality of this review. It has helped us to perform these additional experiments and we are indebted to the reviewers for their insightful comments. The manuscript has been improved tremendously by your efforts, and we are grateful.

Sincerely yours,

Robert E. Carraway, Ph.D.

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University of Massachusetts Medical School
Worcester, MA, 01655

Protein Kinase C Inhibitors Alter Neurotensin Receptor Binding and Function in Prostate Cancer PC3 Cells

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Abstract

Prostate cancer PC3 cells expressed constitutive protein kinase C (PKC) activity that under basal conditions suppressed neurotensin (NT) receptor function. The endogenous PKC activity, assessed using a cell-based PKC substrate phosphorylation assay, was diminished by PKC inhibitors and enhanced by phorbol myristic acid (PMA). Accordingly, PKC inhibitors (staurosporine, Go-6976, Go-6983, Ro-318220, BIS-1, chelerythrine, rottlerin, quercetin) enhanced NT receptor binding and NT-induced inositol phosphate (IP) formation. In contrast, PMA inhibited these functions. The cells expressed conventional PKCs (α, βI) and novel PKCs (δ, ε), and the effects of PKC inhibitors on NT binding were blocked by PKC downregulation. The inhibition of NT binding by PMA was enhanced by okadaic acid and blocked by PKC inhibitors. However, when some PKC inhibitors (rottlerin, BIS-1, Ro-318220, Go-69830, quercetin) were used at higher concentrations (>2μM), they had a different effect characterized by a dramatic increase in NT binding and an inhibition of NT-induced IP formation. The specificity of the agents implicated novel PKCs in this response and indeed, the inhibition of NT-induced IP formation was reproduced by PKCδ or PKCε knockdown. The inhibition of IP formation appeared to be specific to NT since it was not observed in response to bombesin. Scatchard analyses indicated that the PKC-directed agents modulated NT receptor affinity, not receptor number or receptor internalization. These findings suggest that PKC participates in heterologous regulation of NT receptor function by two mechanisms: a)- conventional PKCs inhibit NT receptor binding and signaling; b)- novel PKCs maintain the ability of NT to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NT receptor is also subject to homologous regulation by PKC.

1. Introduction

Neurotensin (NT), a regulatory peptide found in the nervous system and in endocrine cells of the intestinal mucosa [1, 2], has multiple roles as a neurotransmitter and hormone [3-5]. Considerable evidence suggests that NT could contribute to the growth of normal and neoplastic cells [6, 7]. The high affinity G protein-coupled NT receptor NTS1 is overexpressed in many primary human tumors and cell lines, including human prostate cancer PC3 cells [8]. We showed that stimulation of PC3 cells with NT induced a growth response that involved protein kinase C (PKC)-dependent transactivation of the EGF receptor and activation of downstream mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase [9]. The growth effects of NT in other systems and its ability to activate MAPK [10], as well as to enhance cyclic AMP formation [11] and to

phosphorylate GSK-3α/β [12] were also PKC-dependent. In a number of these cells, NT was shown to activate phospholipase C (PLC) [13, 14], stimulating the formation of inositol phosphate (IP) and the mobilization of intracellular Ca^{2+} [15]. Since PKC activity is dependent on the levels of Ca^{2+} and DAG, NT could activate both conventional and novel PKCs, and our recent work indicates that NT enhanced PKC isotype phosphorylation assessed by western blotting in PC3 cells (S Hassan, unpublished results). Not only might PKC mediate some of the effects of NT, but the activation of PKC could possibly exert feedback effects on NT receptor binding and signaling. Despite these findings, there is a paucity of information concerning the relationships between PKC activity and NT receptor function.

The responsiveness of cells to signaling inputs is regulated by a variety of mechanisms that serve to maintain homeostasis and coordinate signaling events. Some of the effects of NT have been shown to display homologous desensitization, such that there is a diminished response to a second stimulus given after the initial one. Heterologous desensitization has also been noted using stimuli that do not activate NT receptors [16]. These findings suggest that NT receptors could be subject to regulation by second messenger-dependent kinases, which could alter the availability of or the activity of the receptor. For the G protein-coupled receptors that have been studied extensively, such as the rhodopsin receptor and the β2-adrenergic receptor, there is considerable evidence suggesting that receptor phosphorylation is an important mechanism for regulating receptor responsiveness [17, 18]. One generality that has emerged from the work in the laboratory of Robert Levkowitz and others is that G protein receptor kinases usually mediate phosphorylation events that are associated with agonist-induced (homologous) receptor desensitization, whereas signaling kinases such as PKC and PKA usually promote receptor phosphorylations involved in heterologous desensitization [18, 19]. Endocytosis by β-arrestin and clathrin dependent mechanisms can also be part of the desensitization process [20]. In regards to NTS1, however, these mechanisms have not been well studied. Agonist dependent phosphorylation of HA-tagged NTS1 has been demonstrated in HEK-293 cells and the importance of certain C-terminal serine residues has been studied [21]. However, the kinases involved and the relationship to the desensitization of NT-induced signaling have not been studied. In addition, the effects on the parameters of receptor binding and internalization have not been defined.

In order to better understand the potential role of PKC in regulating NT receptor function, we set out to determine which PKC isotypes were expressed in PC3 cells and to study the effects of PKC activation and inhibition on NT receptor binding and signaling. The PKC family of serine/threonine kinases phosphorylate

proteins at sites resembling the consensus motif RXXS/TXR [22, 23] and phospho-specific antibodies towards similar motifs have been used to measure PKC substrate phosphorylation in cells [24, 25]. Subcategories of PKC isotypes have been defined, including the conventional PKCs (PKCα, βI, βII and γ), the novel PKCs (PKCδ, ε, θ and η) and the atypical PKCs (PKCζ, μ and ι) based on their activity requirements [26, 27]. Conventional PKCs are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), whereas novel PKCs respond to PS and DAG, and atypical PKCs respond only to PS. Phorbol 12-myristate 13-acetate (PMA), which can mimic the effects of DAG, has been used to activate and/or downregulate conventional and novel isoforms of PKC. The phosphorylation of PKCs and their translocation from cytosol to membrane compartments that occur following acute treatment with PMA have been accepted as indicators of PKC activation. Chronic treatment with PMA on the other hand has generally been found to downregulate PKC expression. Many of the PKC inhibitors that have been used to investigate the involvement of PKC in cellular processes act by blocking the ATP binding site [23]. The most potent of these include the structurally related compounds staurosporine, bisindolylmaleimide I (BIS-1), Ro-328220, Go-6983 and Go-6976 [28]. These compounds exhibit a rather broad isotype specificity [29], except for Go-6976 which preferentially inhibits conventional PKCs, and Go-6983 which does not inhibit PKCμ [30]. Although rottlerin was originally described as a specific PKCδ inhibitor [31], further study indicated that it did not inhibit the enzyme directly [29] and that it could act as a mitochondrial uncoupler [32]. Less potent PKC inhibitors that act by mechanisms that are not well defined include chelerythrine [33] and quercetin [34].

In the work reported here, we first examined the effects of PKC-directed agents on PKC substrate phosphorylation in PC3 cells, establishing that these cells displayed a high level of constitutive PKC activity that responded to PKC activation and inhibition. Then, we investigated the effects of PKC activation, PKC inhibition and PKC isotype knockdown on NT receptor function. Two categories of PKC inhibitors were defined which had dramatically different effects on NT receptor binding and signaling. Based on the selectivity of these inhibitors and the effects of PKC isotype knockdown, we concluded that conventional and novel PKCs played opposite roles in regulating NT receptor function.

2. Materials and methods

2.1 Materials

[^{125}I]-sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]-myo-inositol (60 mCi/mmol) and [methyl-^{3}H]-thymidine were obtained from Perkin Elmer Life Science (Boston, MA). The phospho-specific antiserum to the PKC substrate motif (#2261) was from Cell Signaling Technology (Beverly, MA). The antibodies towards PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCγ and HRP-linked secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal chemiluminescent substrate was obtained from Pierce (Rockford, IL). Staurosporine was from Biomol (Plymouth Meeting PA). Bisindolylmaleimide I (BIS-1), bisindolylmaleimide V (BIS-V), chelerythrine chloride, Go-6976, Go-6983 and Ro-318220 were from Calbiochem (San Diego, CA). Phorbol-12 myristate 13-acetate (PMA), rottlerin, quercetin and all other chemicals were from Sigma (St. Louis, MO).

2.2 Tissue culture

PC3, PC3M, DU145, MatLyLu and HT29 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in F12K medium (PC3, PC3M) and DMEM medium (DU145, MatLyLu, HT29), supplemented with 10% fetal bovine serum [8]. PC3, PC3M (its highly metastatic clone), and DU145 are androgen-independent cell-lines derived from human prostate adenocarcinomas. MatLyLu was derived from a rat prostate carcinoma and HT29 from a human colon carcinoma. LNCaP^{CS} cells, a clone derived from the human prostate LNCaP cell-line, were a gift from Shuk-mei Ho, Department of Environmental Health, University of Cincinnati Medical School. The conditions for growing these cells have been described by us [35]. For western blots, cells were grown in 60 mm dishes; for all other studies, cells were grown in 24 well culture plates. For IP studies, cells were labeled with ^{3}H-inositol using medium 199 (Difco), which has a low inositol content.

2.5 Binding to cultured cells

HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us [13]). In brief, cells in 24-well plates (80-90% confluent) were washed with hepes-buffered Locke-BSA (Locke): 148mM NaCl; 5.6mM KCl, 6.3mM hepes; 2.4mM $NaHCO_3$; 1.0mM $CaCl_2$; 0.8mM $MgCl_2$; 5.6 mM glucose; 0.1% BSA; pH 7.4. Stock solutions (2-10mM) of the PKC inhibitors in dimethyl sulfoxide (DMSO) were stored at -20°C, except for quercetin (prepared just before use), and were diluted in Locke to give ≤1% DMSO. Equilibrium binding at 37°C was performed for 30 min using 10^5 cpm/ml ^{125}I-NT in 1.0 ml Locke. The reaction was stopped on ice, the medium was aspirated and cells were washed in ice-cold saline. Cellular binding was determined as radioactivity per μg protein in cells extracted in 0.3 M NaOH.

Specific binding, displaceable by 1μM NT (20,000-fold excess), was 95% of total binding. Binding displacement curves were constructed and binding parameters were determined as described [35]. Cell surface binding and internalization were assessed by washing cells at room temperature for 2 min with 1 ml of 0.2 M acetic acid, 0.5 M NaCl.

The binding parameters for the ligands used to measure bombesin (BOM) receptor binding and EGF receptor binding in PC3 cells were described by us [13]. In brief, equilibrium binding was performed in a manner identical to that for NT using HPLC-purified ^{125}I-[Nle^{14}]-BOM (10^5 cpm, 50 pM) and ^{125}I-EGF (10^5 cpm, 50 pM). Specific binding was defined as that displaceable by 1 μM BOM (20,000-fold excess) or 0.1 μM EGF (2,000-fold excess).

2.6 IP formation

Formation of [^{3}H]-IP was measured as described by us [13]. Briefly, PC3 cells in 24-well plates were incubated 48 hrs with myo-[^{3}H]-inositol (2.5μCi/ml) in medium 199, 1% fetal calf serum. Cells were pretreated with Locke, 15 mM LiCl for 10 min to inhibit phosphatases. In some cases, they were concomitantly pretreated with test agents or vehicle (DMSO) in Locke, 15mM LiCl as indicated. The reaction was initiated by adding NT, BOM or vehicle (Locke). After 30 min at 37°C, the medium was aspirated, ice-cold 0.1M formic acid in methanol was added and the plates were placed at –20°C overnight. IP was adsorbed to AG-1X8, which was washed in 5mM myo-inositol and eluted in 1.5M ammonium formate. Scintillation counting was performed in Ecoscint (National Diagnostics).

2.7 Inhibition of PKCδ and PKCε in PC3 cells through stable transfection of shRNA expression plasmids

Plasmid constructs (Open Biosystems, Huntsville, AL) designed to constitutively express shRNAs that specifically target either PKCδ (Open Biosystems catalog number RHS1764-9492328 or RHS1764-9098879) or PKCε (RHS1764-9493023) or a control plasmid encoding a non-silencing shRNA (RHS1707) were purchased from the UMASS shRNA Core Facility. The parent plasmid (pSM2a) contains the bacterial puromycin resistance gene under the control of the PGK promoter and the mir-30 gene containing gene-specific synthetic microRNA sequences under the control of the U6 promoter to drive microRNA production [36]. PC3 cells were transfected with individual plasmids on 100 mm tissue culture dishes using FuGene transfection reagent (Roche) according to the manufacturer's instructions, and stably transfected cells were selected by the addition of puromycin (1.0 μg/ml) to the culture medium. Visible clones were isolated after 2-3 weeks of selection using

cloning cylinders, propagated, and screened by western blot analysis to examine PKCδ and PKCε expression levels. Clones displaying significant inhibition of PKCδ or PKCε were grown under continued puromycin selection for use in NT binding and other experiments. PKCδ was significantly inhibited by only one of two shRNAmir constructs tested (RHS1764-9098879).

2.8 Western blotting

Western blot analysis was performed as described by us [9]. In brief, PC3 cells in 60-mm dishes were withdrawn from serum for 24 hrs. Cells, washed in Locke, were exposed to stimuli at 37°C for times indicated. After washing with ice-cold PBS containing phosphatase inhibitors, cells were placed on ice and scraped into 2X SDS buffer (plus inhibitors) and sonicated. Equal amounts of protein were separated by SDS-PAGE using polyacrylamide minigels and electroeluted onto PVDF (Immobilon P, Millipore). Blots were incubated with primary antibodies in blocking buffer for 18 h at 4°C. After washing, blots were incubated with HRP-linked secondary antibodies for 1 h at 20°C, and ECL was performed using supersignal west pico reagent (Pierce, Rockford, IL) and films were scanned for computerized densitometric analysis. After staining with one antibody, blots were stripped and re-probed using different antibodies for comparison and normalization.

3. Results

3.1 PC3 cells displayed constitutive PKC activity and PMA-induced PKC activity

The growth-promoting effects of NT in PC3 cells are PKC-dependent. In order to assess the importance of PKC for NT receptor binding and signaling, we first determined the basal level and stimulated level of PKC activity in the cells by performing western blotting using a phospho-specific antiserum directed towards PKC substrates exhibiting the motif R/K-X-S-Hyd-R/K (where Hyd = hydrophobic amino acid). PKC isotype specificity considerations predicted that this assay would primarily detect substrates phosphorylated by conventional PKCs (α, βI, βII, γ), and might respond to novel PKCε substrates but would react poorly with novel PKCδ substrates [37].

Untreated cells (results not shown), and cells incubated with 0.1% DMSO (vehicle control) for 30 min, exhibited at least ten positive bands with molecular sizes ranging from 40 kDa to 140 kDa (Fig 1A, control). In contrast, cells pretreated for 30 min with 1 μM concentrations of staurosporine, BIS-1, Go-6983 or Ro-318220 displayed less intense labeling of as many as eight of these bands, which was most evident for the 40 kDa, 50

kDa and 140 kDa bands (Fig 1A). The inhibitory effects of Go-6976 and chelerythrine were seen at higher concentrations (5-10 μM), whereas rottlerin (10 μM) and quercetin (15-40 μM) gave only a slight inhibition (Fig 1A and 1C). That these effects were due to the inhibition of constitutive PKC activity was shown by stimulating the cells with PKC activator PMA, which further enhanced the labeling of these bands as well as others not seen under basal conditions (Fig 1B, control). As expected, the effects of PMA were attenuated (Fig 1B and 1D) by pretreating the cells with staurosporine (1 μM), BIS-1 (1 μM), Ro-318220 (1 μM), Go-6976 (1-10 μM), Go-6983 (1-10 μM) or chelerythrine (5 μM). In contrast, rottlerin (1-10 μM) and quercetin (15-40 μM) inhibited only slightly (Fig 1B and 1D). These findings suggested that the identified bands represented PKC substrates which were constitutively phosphorylated by PKCs that were active under basal conditions and that could be further activated by PMA. The effects of the inhibitors were consistent with the prediction that this assay detected primarily substrates that could have been phosphorylated by conventional PKCs (α, βI, βII, γ) or novel PKCε but not novel PKCδ.

3.2 PKC activator PMA inhibited NT binding and NT-induced IP formation in PC3 cells

To determine whether PKC activity could regulate NT receptor function, we first tested the effect of PKC activator PMA on NT binding in intact PC3 cells. Specific binding of ^{125}I-NT (10^5 cpm/ml) to the cells, measured at equilibrium (30 min), was 21.5 ± 1.9 cpm/μg protein (n=9), which was consistent with our prior work [13]. Incubation of the cells with PMA for 15 min decreased NT binding by as much as 42% with an IC50 ≅ 1 nM (Fig 2A). Pretreating the cells with the inhibitor of protein phosphatases (okadaic acid) enhanced the effect of PMA on NT binding (Fig 2B). Pretreating the cells with PKC inhibitors (staurosporine, BIS-1, Go-6983 and Go-6976), using the dose (1 μM) shown in section 3.1 to block the PMA-induced phosphorylation of PKC substrates, prevented the effect of PMA on NT binding (Fig 2C). These results indicated that PMA-induced PKC activity decreased NT binding by an effect involving protein phosphorylation.

Next, we tested the effect of PMA on NT-stimulated PLC activity in PC3 cells. Stimulation of the cells with 30 nM NT (a maximal dose) increased IP formation 3.9 ± 0.4 fold (mean ± SEM, n=8) in keeping with our prior work [13]. As could be predicted from its inhibitory effect on NT binding, PMA diminished NT-induced IP formation and exhibited an IC50 ≅ 1 nM (Fig 4A). Taken together, these results indicated that PMA-induced PKC activity inhibited both NT receptor binding and NT receptor-mediated activation of PLC.

3.3 PKC inhibitors enhanced NT binding and NT-induced IP formation in PC3 cells

The studies in section 3.1 indicated that PC3 cells displayed constitutive PKC activity. To determine whether this constitutive PKC activity regulated NT receptor function, we tested the effects of PKC inhibitors on NT binding and NT-induced IP formation. Six PKC inhibitors (staurosporine, Go-6976, Go-6983, chelerythrine, BIS-1, and Ro-328220) elevated NT binding when used at the low micromolar concentrations shown in section 3.1 to inhibit PKC substrate phosphorylation. Thus, at concentrations ≤2 μM, these agents elevated NT binding by 20-40% (Fig 3A and 3B). NT binding was also elevated 20-40% by rottlerin (0.5-2μM) and quercetin (5-20μM), which inhibited PKC substrate phosphorylation to a lesser degree (Fig 3B and Fig 1C).

In keeping with their ability to enhance NT binding, the PKC inhibitors also enhanced NT-induced IP formation by 15-30% when used in the same concentration range (Fig 4B). The negative control compound for PKC inhibitors (BIS-V) had little effect on NT binding or NT-induced IP formation at concentrations up to 10 μM (results not shown). Taken together, these findings suggested that the ≅30% elevation in NT binding and NT-induced IP formation involved an inhibition of the constitutive PKC activity displayed by these cells. In keeping with this, NT binding was decreased by ≅20% by protein phosphatase inhibitor okadaic acid, which would be expected to enhance the effects of the constitutive PKC activity (Fig 2B).

3.4 Higher doses of some PKC Inhibitors further enhanced NT binding and inhibited NT-induced IP formation

At concentrations >2 μM, five PKC inhibitors (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) dramatically enhanced NT binding (Fig 3A and 3B). Thus, these agents displayed the ability to increase NT binding by as much as 200%. Associated with this response was a dose-dependent inhibition of NT-induced IP formation by as much as 70% (Fig 4C). In contrast, three PKC inhibitors (staurosporine, Go-6976 and chelerythrine) were unable to further elevate NT binding beyond ≅40%, even at higher doses (Fig 3A). In addition, staurosporine and Go-6976 did not inhibit NT-induced IP formation (Fig 4C).

These results indicated that we had defined two categories of PKC inhibitors differing in their ability to modulate NT receptor function. The agents in category I (staurosporine, Go-6976 and chelerythrine) produced only response I (a modest enhancement of NT binding and NT-induced IP formation). In contrast, the agents in category II (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) produced two responses in a dose-dependent manner. At low concentrations response I was obtained, whereas at higher concentrations response II (a dramatic increase in NT binding and an inhibition of NT-induced IP formation) was seen. Three of the agents

in category II were known to inhibit conventional PKCs more than novel PKCs when used at low concentrations [30, 38]. This suggested that our findings might be attributed to a disproportionate inhibition of PKC isotype activity, and we postulated that inhibition of conventional PKCs induced response I, whereas an inhibition of novel PKCs brought about response II. However, not all of the data fit this model and it was still possible that other targets were involved, especially in response II.

3.5 PKC isotype expression and downregulation

PC3 cells expressed conventional PKC subtypes (α, βI) as well as novel PKC subtypes (δ, ε) as determined by western blotting (Fig 5). Pretreating the cells with 1μM PMA for 24 hrs downregulated the expression of conventional PKCα and PKCβI more than novel PKCδ and PKCε (Fig 5). Using this method to downregulate PKC expression, we then tested the effects of PKC inhibitors on NT binding. The results in Fig 3C show that PKC downregulation blocked the modest elevation in NT binding in response to 1μM doses of staurosporine, Go-6976, Go-6983 and Ro-318220, but only reduced (by 25%) the dramatic response to 20μM Ro-318220. These results provided further support for the idea that low doses of PKC inhibitors caused a modest increase in NT binding by inhibiting conventional PKCα and PKCβI, whereas high doses of PKC inhibitors (from category II) caused a dramatic increase in NT binding by inhibiting novel PKCδ and PKCε. Thus, the partial inhibition of the response to 20μM Ro-318220 (Fig 3C) was consistent with the partial downregulation of PKCδ and PKCε (Fig 5). However, it was also possible that targets other than PKC mediated part of this response.

3.6 Effect of PKCδ and PKCε knockdown on NT binding

To determine if the effects of category II inhibitors on NT binding were mediated by PKCδ or PKCε, RNAi technology was used to individually knockdown these novel PKC isotypes. A puromycin resistance gene was incorporated into the DNA construct to facilitate clone selection, and several clones were obtained that displayed >50% knockdown of PKCδ and PKCε (Fig 6A). Based on results from western blotting, one clone representing each treatment was selected for further analysis: nonsense (NS clone); PKCδ(-) clone; and PKCε(-) clone. Basal NT binding, determined when the cells were ≅90% confluent and expressed as cpm/μg protein (mean±SEM, n=4), did not differ for the 3 clones (NS clone, 18.0±1.4; PKCδ(-) clone, 15.8±1.2; and PKCε(-) clone, 15.4±1.1). Assessing the effects of BIS-1 and rottlerin on cellular NT binding in these clones indicated that they were also not significantly different (Table 1). These results indicated that PKCδ knockdown or PKCε

knockdown by itself was not sufficient to alter basal NT binding or the effects of BIS-1 and rottlerin on NT binding. One possible explanation was that knockdown of both PKCδ and PKCε (and perhaps PKCα and PKCβI) was necessary for the effect.

3.7 Effect of PKCδ and PKCε knockdown on NT-induced IP formation

NT was less effective in elevating IP formation in the PKCδ(-) and the PKCε(-) clones as compared to the NS clone (Fig 6B). This was not due to a general effect on PLC activity or the level of phospholipid substrate since the IP response to 3nM BOM was not inhibited in the PKCδ(-) clone (% control response, 95±2; n=6) and was enhanced in the PKCε(-) clone (% control response, 148±13; n=6). Consistent with the importance of both PKCδ and PKCε in this regulation, BIS-1 further inhibited the NT response and further enhanced the BOM response, even in the PKCδ(-) and PKCε(-) clones (Fig 6C). These results were in keeping with the hypothesis that PKCδ and PKCε activity maintained the ability of NT receptor to stimulate PLC.

3.8 PMA decreased and PKC inhibitors increased both cell-surface NT binding and internalization in PC3 cells

Since internalization of the NT receptor occurs during NT binding to PC3 cells [13], we sought to determine if activation or inhibition of PKC altered this process. NT receptor internalization, determined as the percentage of NT binding that resisted acid washing of the cells, was 69±3% (mean ± SEM, n = 5) under control conditions. Pretreatment of the cells with PKC activator PMA decreased both cell-surface NT binding and internalized NT binding by ≅50% (Fig 7A). Although there was a tendency for PMA to increase the percentage of internalization (Fig 7A legend), the results did not differ significantly (e.g., control, 68±2% vs 25 nM PMA, 72±2%; $p > 0.1$).

Pretreatment of the cells with PKC inhibitor BIS-1 increased both cell-surface NT binding and internalized NT binding by as much as 2.5-fold (Fig 7B). There was a tendency for BIS-1 to decrease the percentage of internalization (Fig 7B legend); however, the results did not differ significantly (e.g., control, 69±3% vs 1 μM BIS-1, 64±2%; $p > 0.05$). These results indicated that the changes in NT binding induced by PKC activation or PKC inhibition could not be attributed solely to any effects on NT receptor internalization.

3.9 Clathrin-coated vesicle-mediated Endocytosis

To assess the importance of clathrin-coated vesicle-mediated endocytosis in the effects of PKC inhibitors, cellular NT binding was measured in hypertonic medium (Locke containing sucrose), which is known to block clathrin-coated vesicle-mediated endocytosis of ligands and receptors [39]. Sucrose dose-dependently inhibited

NT binding to the cells, with 0.33 M sucrose giving about 45% inhibition (Fig 8A). However, sucrose unexpectedly inhibited both NT binding to the cell-surface and NT internalization, such that the internalized percentage was only slightly decreased (Fig 8B). This was in contrast to the results for BOM receptor which was used as the positive control [40], where sucrose inhibited BOM internalization, enhanced BOM cell-surface binding and decreased the internalized percentage (Fig 8B). In addition, when the NT binding reaction was done in 0.33 M sucrose, the response to BIS-1 was enhanced and the effect of PMA was diminished (Table 2). These findings suggested that hyperosmolar sucrose stimulated PKC activity and indeed, we found that cellular PKC substrate phosphorylation was enhanced by exposing the cells to 0.33 M sucrose for 15 min (Fig 8C).

We interpreted these findings to indicate that clathrin-coated vesicle-mediated endocytosis participated in NT receptor internalization, but the effect of BIS-1 on NT receptor binding persisted despite inhibition of this process with sucrose. Furthermore, our results suggested that sucrose decreased cellular NT binding at least partly by activating PKC, which is consistent with work in NIH/3T3 cells showing that hyperosmolality increased PKC activity within 10 min [41].

4.0 PMA increased and PKC inhibitor decreased the IC50 for NT binding in PC3 cells

To determine whether the effects of PKC-directed agents on NT binding reflected changes in receptor affinity or receptor number, NT displacement data were analyzed. The PKC activator PMA shifted the NT displacement curve to the right (Fig 9A). The average IC50 (mean ± SEM) was increased from 1.07±0.05 nM (control) to 2.17±0.17 nM (0.5μM PMA; $p<0.01$) in 3 experiments. In contrast, the PKC inhibitor Ro-318220 shifted the NT displacement curve to the left (Fig 9B). The average IC50 was decreased from 1.35±0.22 nM (control) to 0.55±0.09 nM (1μM Ro-318220; $p<0.01$) in 3 experiments. Scatchard analyses (Fig 9C and 9D) indicated that the changes in NT binding were due to the demonstrated changes in receptor affinity and could not be attributed to changes in receptor number. Studies comparing the effects of inhibitors from category I and II showed that 1μM Go-6976 gave results that were similar to those for 1μM Ro-318220 (results not shown). In addition, Fig 9E shows that the effect of the high dose (20μM) of the category II inhibitor rottlerin on the binding parameters was similar to that of the low dose (1μM), except that there was a greater shift in the Ki to even higher affinity, without an increase in receptor number.

4.1 Receptor specificity

To determine if these responses were specific to the NT receptor, we tested PKC inhibitors for effects on BOM receptor binding and EGF receptor binding in PC3 cells [13]. Since the BOM [42] and EGF receptors [43] are known to be regulated by PKC, this provided another test regarding the specificity of these agents. Under control conditions, BOM receptor binding was 112±14 cpm/μg (n=6) and EGF receptor binding was 35.7±2.7 cpm/μg (n=6) in agreement with our prior work. Pretreatment of the cells with PKC inhibitors from category I (staurosporine) and category II (BIS-1 and rottlerin) modestly elevated BOM receptor binding (<20% increase) and EGF receptor binding (<30% increase). These agents were effective in the low micromolar range and their effects did not increase dramatically at higher doses. These results indicated that the robust elevation (>200% increase) in NT receptor binding caused by category II PKC inhibitors was specific to the NT receptor, whereas the smaller elevation (<30%) was also seen for the BOM and EGF receptors.

The PKC-directed agents were also tested for the ability to alter IP formation in response to BOM since it was known to stimulate PLC [13]. IP formation in PC3 cells was enhanced 4.7±1.1 fold by 1 nM BOM and 14.5±2.5 by 10 nM BOM. Therefore, we used 2 nM BOM as the stimulus to test the effects of the agents. As expected, the PKC activator PMA inhibited the response to BOM, exhibiting an IC50 near 1 nM (Table 3). When used at nanomolar and low micromolar doses, the PKC inhibitors (staurosporine, Go-6976, Go-6983, BIS-1, Ro318220 and rottlerin) enhanced BOM-induced IP formation by 20-40% (Table 3). These results, which were similar to those for NT, suggested that the constitutive PKC activity in PC3 cells inhibited PLC activation in response to both NT and BOM. This interpretation is consistent with work showing that BOM receptor function is subject to PKC regulation [44].

Although the enhancement of the IP response to BOM fell off at higher doses of rottlerin (Table 3) as was seen for NT (Fig 4C), in general the results for BOM differed from those for NT. For example, doses of BIS-1, Ro-318220 and Go-6983 that inhibited NT-induced IP formation by >40% (Fig 4C) did not inhibit BOM-induced IP formation (Table 3). These results indicated that the inhibition of NT-induced IP formation by the PKC inhibitors in category II was relatively specific to NT.

4.1 Cell specificity

To determine if the effects of PKC inhibitors were specific to PC3 cells, we assessed the effect of BIS-1 on NT binding in other cancer cell lines, including prostate carcinomas (PC3M, DU145, LNCaP^{CS}, MatLyLu) and a colon carcinoma (HT29). The level of NT receptor expression in these cell lines was previously

described by us [35]. As compared to the vehicle control (0.06% DMSO), specific binding in the presence of 6 μM BIS-1 was (mean ± SEM; n=3 experiments) was: PC3 (186±11); HT29 (188±12); MatLyLu (217±13); PC3M (227±12); $LNCaP^{CS}$ (162±15) and DU145 (276±18). These results indicate that 6 μM BIS-1 enhanced NT binding similarly in a number of cancer cell lines.

4. Discussion

Here, we report for the first time that NT receptor function in a number of prostate cancer cell lines is regulated by endogenous PKC activity. Focusing on PC3 cells, we demonstrated the expression of constitutive PKC activity that, under basal conditions, suppressed NT receptor binding and NT-induced IP formation. Thus, eight different PKC inhibitors (staurosporine, BIS-1, Ro-318220, Go-6976, Go-6983, chelerythrine, rottlerin and quercetin) enhanced NT binding and NT-induced IP formation by 20-40% at low micromolar concentrations that for most of these agents were shown to inhibit conventional PKC substrate phosphoryation in the cells. Not only was NT receptor function inhibited in a tonic manner by the basal PKC activity but pharmacologic activation of PKC was also shown to further inhibit NT receptor function. Thus, PKC activator PMA further inhibited NT binding and NT-induced IP formation. Our results established that NT receptor function was regulated by a heterologous desensitization process that appeared to involve primarily conventional PKC activity. However, our studies also uncovered a second mechanism by which novel PKC activity appeared to maintain (perhaps via effects on G protein coupling) the ability of NT receptor to activate PLC. Overall, our findings imply that, depending on the predominant PKC isotypes involved, heterologous regulation of NT receptor function can serve to either inhibit or maintain NT receptor function. Since NT-induced growth signaling in PC3 cells is PKC-dependent [9] and is associated with enhanced PKC isotype phosphorylation (S Hassan, unpublished data), our findings are also compatible with the existence of homologous regulation of NT receptor function via feedback effects of the PKC isotypes activated by NT. However, further studies are needed before a definitive conclusion can be drawn.

Although we have not yet identified all of the PKC isotypes involved, our studies point towards roles for both conventional (Ca^{2+}-sensitive) PKCs and novel (Ca^{2+}-insensitive) PKCs, with the latter exerting more striking effects. This was suggested initially by the fact that rottlerin, a PKCδ (novel)-specific inhibitor, was one of the most potent and efficacious agents to enhance NT receptor binding (Fig 3B), whereas the conventional-specific inhibitor Go-6976 was much less effective (Fig 3A). However, the results were puzzling

since some of the inhibitors with broad specificity (BIS-1, Ro-318220, Go-6983) were quite effective while others (staurosporine, cherlerythrine) were rather ineffective (Fig 3A and 3B). Furthermore, the effects of some of the agents on NT-induced IP formation were dose-dependently biphasic, while others were monophasic (Fig 4B and 4C). Careful analysis finally led us to conclude that there were two different responses: response I was characterized by a modest enhancement (≅40%) in NT receptor binding and NT-induced IP formation; response II involved a dramatic enhancement (≅200%) in NT receptor binding associated with an inhibition of NT-induced IP formation. Response I was brought about by most of the PKC inhibitors at the low micromolar concentrations that were shown to inhibit conventional PKC substrate phosphorylation. On the other hand, response II could only be induced by inhibitors in category II (rottlerin, BIS-1, Ro-318220, Go-6983 and quercetin) and only at higher concentrations (>2μM).

It seems probable that PKC inhibition was involved in both response I and II, and that the outcome depended on the differential effects on specific PKC isotypes. Western blotting demonstrated that the major isotypes present were the conventional PKCs (α, βI) and the novel PKCs (δ, ε). The results for Go-6976 (specific for conventional PKCs) were consistent with the idea that inhibition of PKCα and/or PKCβI produced response I. The results for BIS-1 and Ro-318220 (known to require 5-16 fold higher concentrations to inhibit novel PKCs) and for rottlerin (putative PKCδ inhibitor) suggested that response II involved an inhibition of PKCδ and/or PKCε. To explain the results for the other inhibitors (which had broad specificity), one might propose that staurosporine and chelerythrine were unable to gain access to or sufficiently inhibit PKCδ and/or PKCε and thus, did not produce response II. Consistent with this interpretation, we found that knockdown of PKCδ or PKCε reproduced at least one aspect of response II, the inhibition of NT-induced IP formation. Although the PKCδ(-) and PKCε(-) clones did not exhibit the enhanced NT binding that is characteristic of response II, this apparent difference in PKC isotype dependence could have been due to differences in the assay conditions. For example, the NT binding assay (in contrast to the IP assay) did not involve stimulating the cells with high levels of NT that might have induced homologous desensitization. Another explanation is that knockdown of both PKCδ and PKCε (and possibly PKCα and PKCβI) might have been required to reproduce the effects of category II inhibitors on NT binding. Finally, it might have been that some of the effects of category II inhibitors on NT binding involved targets other than PKC.

The simplest interpretation of our results at this time is that inhibition of conventional PKCα and PKCβI produced response I, whereas the additional inhibition of novel PKCδ and PKCε produced response II. Thus, when BIS-1 was used at low doses that preferentially inhibited conventional PKCs, NT binding and IP formation were modestly enhanced. It seems likely that the increase in NT receptor binding led to the enhanced IP response. Therefore, a reasonable model is that conventional PKCs modulate NT binding to regulate receptor function. On the other hand, when BIS-1 was used at higher doses that inhibited novel as well as conventional PKCs, NT binding was dramatically enhanced and IP formation was inhibited. Since the inhibition of IP formation was specific to the NT receptor, it seems likely that this involved some form of G protein uncoupling. For example, novel PKC activity might maintain NT receptor-effector function by keeping G protein receptor kinases in check. Whatever the mechanism, this model is consistent with most of the data. For example, knockdown of PKCδ or PKCε would be expected to inhibit NT-induced IP formation but it would not necessarily cause the dramatic increase in NT receptor binding. This is because the uncoupled receptor would still be subject to binding inhibition imposed by constitutive PKCα and PKCβI activity.

The mechanistic studies reported here show that the modulation of NT receptor binding by the PKC-directed agents could not be attributed to changes in receptor number or receptor internalization. However, PKC activation or inhibition induced a remarkable change in receptor affinity that was consistent with the idea that PKC activity shifted the NT receptor to a low affinity state. Thus by blocking the constitutive PKC activity of the cells, PKC inhibitors shifted the NT receptor to a higher affinity state resulting in increased binding activity. Consistent with this, NT binding to the cell-surface and NT internalization were increased similarly and dose-responsively by BIS-1. The enhancing effects of BIS-1 persisted despite the inhibition of clathrin-coated vesicle-mediated endocytosis by sucrose, indicating that the mechanism was not likely to involve a change in receptor trafficking. Although it seems clear that a shift in NT receptor affinity is the basis for the regulation of NT binding by PKC, we do not know if the effects of PKC are direct or indirect. NT has been shown to induce phosphorylation of HA-tagged NT receptor in HEK-293 cells [21], but it is not known whether PKC participated in this or if PKC can under any conditions phosphorylate NT receptor. Since PKC inhibitors had only modest effects on BOM receptor binding and EGF receptor binding, the dramatic effects on the behavior of the NT receptor appeared to be receptor-specific. We demonstrated the effect of BIS-1 on NT receptor binding in a number of cancer cell lines, indicating that this behavior may apply generally to cells expressing the NT receptor.

It is interesting to note that the ability to alter NT receptor function was related to the chemical structures of the PKC inhibitors that were analogs of staurosporine. The agents in category II that induced dramatic effects on NT binding (BIS-1, Go-6983 and Ro-318220) were derivatives of maleimide, whereas the agents in category I that induced moderate effects on NT binding (staurosporine and Go-6976) were derivatives of carbazole. It is tempting to speculate about how the chemical nature of these derivatives might relate to their differing effects on NT receptor function. In comparison to the carbazoles, the maleimides offer a more highly conjugated system that could promote redox reactions, one possibility involving donation of the central imide hydrogen. Interestingly, the maleimide derivative with a CH_3 group instead of hydrogen at this position (BIS-V) was inactive as a PKC inhibitor [45] and unable to modulate NT receptor function. This suggested that an antioxidative redox effect could play a role in these effects. We found that antioxidative polyphenols that can accumulate in membranes (and might possibly inhibit PKC) induced the type II response in PC3 cells [46]. BIS-1 is known to accumulate in mitochondrial membranes [47]; PKCδ was shown to translocate to mitochondrial membranes during apoptosis [48, 49]; and Ro-318220 was found to induce mitochondrial apoptosis [50]. We showed that inhibitors of mitochondrial oxidative phosphorylation induced the type II response in PC3 cells [45]. Superoxide and hydrogen peroxide, which are produced by mitochondria, were found to activate PKC and this effect was reversed by antioxidants [51]. These findings suggest that the type II response could involve antioxidative effects of these agents on PKCδ and/or PKCε within mitochondria or other targets in PC3 cells.

Given that PKC activity can vary depending on cell type, stage of development and environmental inputs, these findings underline the importance of controlling these conditions when assessing NT receptor function in different systems. Since NT is an important mitogen in cancer cells, our findings suggest that the PKC-directed agents in category II, which inhibit NT receptor signaling, could be useful lead compounds for the development of new anticancer drugs. If these compounds produced their effects on NT receptor function by targeting PKCδ and/or PKCε, then these NT assays might be used to screen for substances that specifically inhibit or activate these novel PKCs. This could be useful since PKCδ and PKCε play important roles in cell cycle regulation [52], growth stimulation [53], growth inhibition [54] and apoptosis [55]. It should be noted that androgens can regulate the activity of the NT system [56, 57] as well as the expression of PKCδ in prostate LNCaP cells [58].

In conclusion, constitutive PKC activity was demonstrated in PC3 cells, which under basal conditions caused an inhibition of NT receptor function. Accordingly, low micromolar doses of PKC inhibitors released

NT receptor from this inhibitory influence, enhancing NT binding and NT-induced IP formation moderately. This type I response appeared to involve inhibition of the conventional PKCs (α, βI). In addition, a second response was identified, which was induced by higher concentrations of a select group of PKC inhibitors. This type II response, characterized by a dramatic increase in NT binding and a pronounced inhibition of NT-induced IP formation, appeared to involve the novel PKCs (δ, ε), although other targets were possible. Overall, the results were consistent with the hypothesis that NT receptor function was subject to heterologous regulation by PKC and that two distinct mechanisms were involved.

Table 1

Effect of BIS-1 and Rottlerin on NT Receptor Binding in PKC Knockdown and Control Clones of PC3 Cells

Cell Clone	NT Binding (% DMSO control)			
	BIS-1		Rottlerin	
	0.5μM	2.5μM	0.5μM	2.5μM
NS control	155±14	216±20	150±16	181±17
PKCδ (-)	168±13	254±17	145±13	222±18
NS control	140±13	225±19	140±14	236±19
PKCε (-)	167±12	268±21	177±15	244±18

NT receptor binding to each PC3 cell clone was measured in the presence of BIS-1 or rottlerin at the indicated concentrations as compared to the vehicle (DMSO) control. The cells (80-90% confluent) were preincubated 15 min with the agents in Locke prior to the binding reaction. Control NT binding for each clone was similar when expressed as cpm/μg protein: NS control, 18.0±1.4; PKCδ (-); 15.8±1.2; PKCε (-), 15.5±1.1 (n=4). For each clone, the effects of BIS-1 and rottlerin were expressed as % control NT binding (mean±SEM; n=3 experiments). The results for the different clones were not significantly different.

Table 2

Effects of PKC-directed Agents on Internalized and Cell-surface NT Binding in Locke and Hypertonic Sucrose

Agent	Concentration (μM)	Internalized NT Binding (% control)[a]		Cell-surface NT Binding (%control)[a]	
		Locke	sucrose	Locke	sucrose
DMSO	(0.1%)	100	100	100	100
BIS-1	0.6	126±4	153±5 [b]	117±4	141±4 [b]
	3.0	157±13	216±18 [b]	145±6	194±10 [b]
PMA	1	70±6	95±5 [b]	48±6	77±11 [b]

[a] The effect of each agent on NT receptor binding was tested in Locke or in Locke containing 0.33 M sucrose. The cells (80-90% confluent) were pre-incubated 15 min with the agents or the vehicle control (0.1% DMSO) in Locke prior to the binding reaction. The medium was changed to Locke or sucrose containing the agents, and ^{125}I-NT was added. After 30 min, the cells were placed on ice and NT internalization and NT binding to the cell-surface were measured using the acid washing technique. The data (mean±SEM; n=4 experiments) were expressed relative to the appropriate vehicle control. For the control condition, sucrose reduced internalized NT binding and cell-surface NT binding to a similar extent (45-55%).

[b] Results in sucrose differed significantly from those in Locke ($p<0.05$).

Table 3. Effects of PKC-directed Agents on BOM-induced IP Formation in PC3 Cells

agent	dose (μM)	IP formation [a] (% control)
PMA	0.001	43 ± 4 [c]
	0.04	25 ± 3 [c]
	0.1	18 ± 2 [c]
staurosporine	0.2	129 ± 7 [b]
	0.8	120 ± 5
	3.2	117 ± 7
Go-6976	0.4	138 ± 5 [b]
	2	107 ± 5
	10	109 ± 8
Go-6983	0.4	129 ± 6 [b]
	2	129 ± 6 [b]
	10	119 ± 5
BIS-1	0.1	117 ± 5
	0.5	124 ± 5 [b]
	2.5	138 ± 5 [b]
Ro-318220	0.2	125 ± 6
	0.8	129 ± 6 [b]
	3.2	78 ± 6
rottlerin	0.3	146 ± 9 [b]
	1	81 ± 6
	10	45 ± 6 [c]

[a] Cells were pretreated with the indicated concentrations of each agent or vehicle control for 15 min, then stimulated with 2 nM BOM for 30 min, and IP formation was measured. The increment in IP formation was expressed as % control (mean ± SEM) for at least three experiments.

[b] BOM-induced IP formation was significantly increased ($p<0.05$).

[c] BOM-induced IP formation was significantly decreased ($p<0.01$).

Figure legends

Fig 1. PC3 cells displayed constitutive PKC activity (A and C) and PMA-induced PKC activity (B and D) that was sensitive to PKC inhibitors. Endogenous PKC substrate phosphorylation was assessed by western blotting using a phopho-specific antiserum to the motif R/K-X-S-Hyd-R/K. In A, quiescent cells were incubated with staurosporine (1 μM), BIS-1 (1 μM), Go-6976 (1 μM), Go-6983 (1 μM), Ro-318220 (1 μM), chelerythrine (5 μM), quercetin (15 μM) rottlerin (1 μM), or 0.1% DMSO (control) for 30 min. In B, cells pretreated as in A were then stimulated with 100 nM PMA for 5 min. In C, quiescent cells were incubated with quercetin (40 μM), rottlerin (10 μM), Go-6976 (10 μM), Go-6983 (10 μM), or 0.1% DMSO (control) for 30 min. In D, cell pretreated as in C were then stimulated with 100 nM PMA for 5 min. Cell extracts containing equal amounts of protein were subjected to western blotting using the PKC substrate-specific antiserum. Shown are typical results that are representative of at least three experiments each.

Fig 2. PKC activator PMA decreased NT binding to PC3 cells (A), and its effect was enhanced by protein phosphatase inhibitor okadaic acid (B) and inhibited by pretreatment with PKC inhibitors (C). Specific NT binding was measured to intact cells at 37° C. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to measuring NT binding. In B, cells were pretreated with okadaic acid in the presence and absence of 100 nM PMA for 20 min prior to measuring NT binding. In C, cells were pretreated with staurosporine (1 μM), BIS-1 (1 μM), Go-6983 (1 μM), Go6976 (1 μM) or 0.05% DMSO (vehicle control) for 20 min. Then, 10 nM PMA was added for 10 min and NT binding was measured. For each experiment, the data (mean ± SEM) are representative of at least three experiments.

Fig 3. PKC inhibitors enhanced NT binding to PC3 cells (A, B), and the response to low doses of PKC inhibitors was blocked by downregulation of PKC (C). Specific NT binding was measured to intact cells. In A and B, sub-confluent cells were pretreated with the indicated concentrations of agents for 15 min prior to measuring NT binding. The data (mean ± SEM) were from at least 4 experiments. In C, sub-confluent cells were pretreated with 1μM PMA (downregulated) or 0.01% ethanol (control) for 24 hrs. After washing in Locke, cells were pretreated with staurosporine (ST), Go-6976 (Go-76), Go-6983 (Go-83), Ro-318220 (Ro-31) or 0.05% DMSO (CON) for 15 min prior to measuring NT binding. The data (mean ± SEM; n=4) from two experiments were expressed as % control. Note that PKC downregulation blocked

the response to the low dose (1 μM) of each inhibitor and diminished the response to the high dose (20 μM) of Ro-31220.

Fig 4. NT-induced IP formation was inhibited by PKC activator PMA (A), whereas it was enhanced by low doses (B) and inhibited by high doses (C) of PKC inhibitors. IP formation was measured in response to 30 nM NT. The effect of NT (≅4-fold elevation) was calculated as the increment above the appropriate control. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to stimulation with NT. In B and C, cells were pretreated with the indicated agents for 15 min prior to stimulation with NT. For each panel, the data (mean ± SEM) are representative of at least three experiments.

Fig 5. Western blot assessing the expression of PKC isotypes in control PC3 cells and in cells pretreated with PMA. Cells grown in 60mm dishes were withdrawn from serum for 24 hrs and then pretreated with 0.1% DMSO or 1 μM PMA for 24 hrs. Whole cell extracts were prepared, 40μg protein was subjected to SDS-PAGE and western blotting was performed using antisera specific for PKCα, PKCβI, PKCβII, PKCγ, PKCδ and PKCε. β-actin was used as the loading control. The results shown are representative of two experiments.

Fig 6. Effect of PKCδ and PKCε knockdown on PKC expression (A), NT-induced IP formation (B) and on the ability of BIS-1 to alter NT-induced and BOM-induced IP formation (C). In A, an equal number of cells from each clone was extracted, 40μg protein was subjected to SDS-PAGE and western blotting was performed for PKCδ and PKCε. β-actin was the loading control. The results, representative of multiple experiments using 2 to 5 clones each, indicate >50% knockdown of PKCδ and PKCε. In B, equal numbers of cells from each clone were stimulated with the indicated doses of NT. IP formation was measured and the responses were calculated as % maximal. In C, cells pretreated 15 min with 2.5μM BIS-1 or vehicle control, were stimulated with 10 nM NT or 3 nM BOM. The IP responses were calculated as % control, which gave ≅4-fold elevation (NT) and ≅10-fold elevation (BOM). In B and C, the data (mean ± SEM) are representative of at least three experiments.

Fig 7. NT binding to PC3 cells was diminished by PMA (A) and enhanced by BIS-1 (B), without an effect on the percentage of ^{125}I-NT internalized by the cells. Cells were pretreated 10 min with indicated

concentrations of PMA, BIS-1 or vehicle control. After the binding reaction, cell-surface and internalized binding were determined by acid washing. The data (mean ± SEM) were pooled from three experiments. In A, internalization expressed as percentage of total binding (mean ± SEM) was: 68±2, 70±2, 70±2, 72±2, 68±2 and 71±2 at the 0, 1, 5, 25, 125 and 625 nM doses of PMA. In B, % internalization was 69±3, 66±2, 65±2, 64±2, 64±2 and 69±2 at the 0, 0.07, 0.25, 1, 4 and 20 μM doses of BIS-1.

Fig 8. Effect of hypertonic sucrose on NT receptor binding (A), NT receptor and BOM receptor internalization (B) and PKC substrate phosphoryation (C) in PC3 cells. In A, total cellular NT binding was measured in Locke containing varying concentrations of sucrose and the results were expressed relative to control. In B, the effect of 0.33 M sucrose on cell-surface and internalized binding for the NT receptor and the BOM receptor is shown. After binding was performed with ^{125}I-NT or ^{125}I-BOM, internalized and cell-surface radioactivity was measured by acid washing. The data (mean±SEM) show % binding obtained in sucrose relative to Locke control. Internalization of NT receptor expressed as percentage of total binding was 70±2 (Locke) and 65±2 (sucrose), while that for the BOM receptor was 68±1 (Locke) and 54±1 (sucrose). In C, quiescent cells, pretreated with 1μM BIS-1 or vehicle control for 30 min, were stimulated with Locke or 0.33 M sucrose for 15 min. Cell lysates were subjected to Western blotting using the PKC substrate specific antiserum. β-actin was used as loading control. Shown are typical results representing 3 experiments.

Fig 9. Binding displacement curves (A, B) and Scatchard plots (C, D, E) for NT binding to PC3 cells in presence and absence of PKC-directed agents. Cells were pretreated with agents indicated or control for 30 min. Then, specific NT binding was measured in the presence of indicated concentrations of NT at equilibrium (30 min). NT binding was decreased 35% by 100 nM PMA, whereas it was increased 54% by 1 μM Ro-318220. In A and B, log dose-response plots are shown in which NT binding was expressed as percentage of control. The IC50 was shifted to the right by PMA (A) and to the left by Ro-318220 and rottlerin (B). Results are from typical experiments that were repeated twice. C, D and E are Scatchard plots for typical experiments, showing that the changes in NT binding were due to shifts in receptor affinity (slope of line) rather than receptor number (intercept at x-axis). In C, note that two components were seen for PMA and the line drawn estimates only the high affinity sites. In C, Ki was 1.0 nM (control) and 2.1 nM (PMA); Bmax was 260 fmol/mg (control) and 320 fmol/mg (PMA). In D, Ki was 1.0 nM (control) and 0.42 nM (1μM Ro-318220); Bmax was 203 fmol/mg (control) and 186 fmol/mg (1μM Ro-31882). In E, Ki

was 1.2 nM (control), 0.69 nM (1μM rottlerin) and 0.23 nM (20μM rottlerin); Bmax was 246 fmol/mg (control), 219 fmol/mg (1μM rottlerin) and 199 fmol/mg (20μM rottlerin).

Ackowledgements

This work was supported by Department of Defense (DOD) Grant PC050212, although the opinions expressed are solely the responsibility of the authors and not necessarily those of the DOD.

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*** Response to Reviewers Comments**

Dr. Juris J. Meier, M.D., Associate Editor
Dr. Wolfgang E. Schmidt, M.D., Ph.D., Editor in Chief
Regulatory Peptides November 30, 2007

Dear Editors and Manuscript Reviewers: RE: REGPEP-D-07-00039

With this letter, we are submitting a revision of the above referenced manuscript, which upon initial review was considered suitable for publication in Regulatory Peptides contingent on revision according to the reviewers' comments. We have addressed all of the issues raised by the reviewers and complied with many of the reviewers' suggestions. Below we list each of the points made by the reviewer, and then summarize and discuss each of our responses.

Reviewer Comment #1- In short the implication is that neurotensin can activate PKC and dependent upon the predominant PKC family member, this can serve to inhibit or potentiate further the response to neurotensin.

Response - Although we did mention in the final sentence of the original Abstract that "homologous" regulation of NT receptor was possible, our main point was that the results of the current study indicate "heterologous" regulation of NT receptor by constitutively active PKC. It may be important to distinguish these two since most of the current study focuses on PKC activity that is evident under basal conditions and in response to activator PMA but not in response to NT stimulation. However the reviewer raises a very important issue here which is difficult to resolve. The traditional view of receptor binding is that the very low concentration of ^{125}I-labeled NT ligand used does not activate or desensitize a significant number of receptors, and therefore, the measurements of affinity and receptor number reflect the unstimulated state. However, it is conceivable (in my mind) that the "basal" binding parameters actually reflect a desensitized state that comes about by the local activation of the receptors that are hit with the labeled ligand and the local feedback effect of activated PKC. In other words, the binding enhancement by PKC inhibitor could reflect an inhibition of constitutively active PKC or an inhibition of the feedback process. At the present time, we have chosen the former interpretation since the PKC substrate phosphorylation assay shows that the cells display "basal" activity in the absence of NT or other PKC activators, presumably from constitutively active PKC. We also do not discuss this point since we have no evidence to challenge the current dogma regarding the interpretation of binding data.

Reviewer Comment #2 - I would like to see an attempt to knockdown PKC delta using an RNAi approach to test their proposed mechanism more directly. This can in principle be extended to the other PKC family members.

Response - Using puromycin to facilitate clone selection, we have now successfully obtained several clones exhibiting >50% knockdown of one of the novel PKC subtypes (PKC delta or PKC epsilon) as well as clones from scrambled RNAi controls. We have examined the behavior of these clones in the NT binding assay (results are presented in 3.6 of the revised manuscript) and in the IP assay (results presented in 3.7 of revised manuscript). Our studies show that knockdown of either PKC delta or PKC epsilon did not alter basal NT binding and did not change the response to category II PKC inhibitors (Table 1, revised manuscript). However, these knockdown clones displayed a decreased ability to activate PLC in response to NT (Fig 6, revised). These results indicated that knockdown of PKC delta or PKC epsilon by itself was not sufficient to negate the effects of these PKC inhibitors on NT binding, supporting the idea that several PKCs (most likely conventional PKC alpha and PKC beta) regulate NT binding. On the other hand, the IP results support a role for PKC delta and PKC epsilon in regulating receptor-effector coupling. These results are presented, interpreted and extensively discussed in the sections of the revised manuscript that are highlighted. In short, we are very pleased that the reviewer suggest these experiments since the results support our current model, which is that conventional PKCs regulate NT binding affinity whereas novel PKCs regulate receptor-effector coupling. The differential

effects of the various PKC inhibitors can be largely explained on the basis of their ability to dose-dependently and disproportionately inhibit these PKC isotypes.

Reviewer Comment #3- In order to comprehensively rule out the possibility that these effect are mediated through alterations in the trafficking/internalization of receptors I would like to see the assays repeated in combination with a panel of dominant negative constructs targeting endocytic trafficking.

Response- We have addressed this point and our results presented in section 3.9 and Fig 8 (revised manuscript) indicate that inhibition of clathrin-coated vesicle-mediated endocytosis using sucrose did not alter the effects of BIS-1on NT cell-surface binding or on NT internalization. BIS-1 worked despite blocking clathrin-coated vesicle endocytosis. The fact that sucrose inhibited clathrin-coated internalization in PC3 cells was shown using the bombesin receptor as a positive control (known to use this pathway). These results support our other results (Fig 7) showing that BIS-1 and PMA alter both cell-surface and internal binding, and (Fig 9) showing that BIS-1 and PMA modulate NT receptor affinity. None of these agents alter the internalization expressed as percentage of total binding. Thus, the evidence is overwhelming that PKC inhibitors enhance NT binding by an effect on receptor affinity that translates into more binding to the cell surface and as a result more internalization, but the internalization process itself does not appear to be modulated by BIS-1. Also, bombesin binding was not much affected by BIS-1 and bombesin internalization was not enhanced by BIS-1.

Comments to Editor and reviewers- We are very pleased with the quality of this review. It has helped us to perform these additional experiments and we are indebted to the reviewers for their insightful comments. The manuscript has been improved tremendously by your efforts, and we are grateful.

Sincerely yours,

Robert E. Carraway, Ph.D.

Professor of Physiology
University of Massachusetts Medical School
Worcester, MA, 01655

Figure

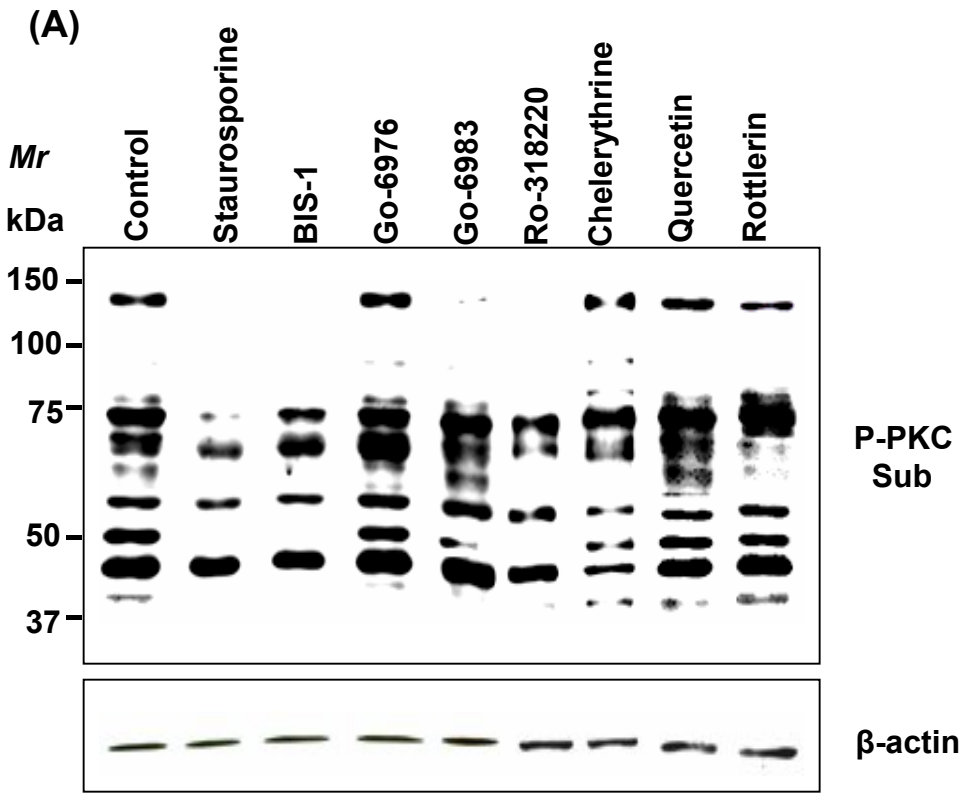
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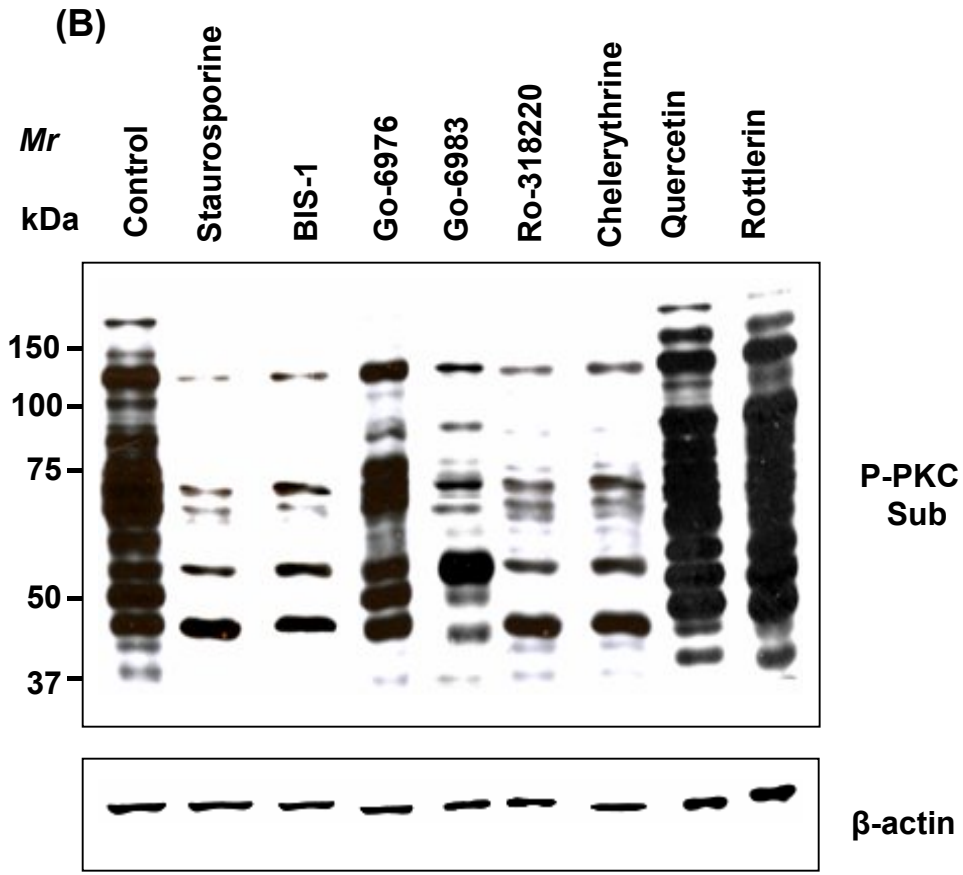
Figure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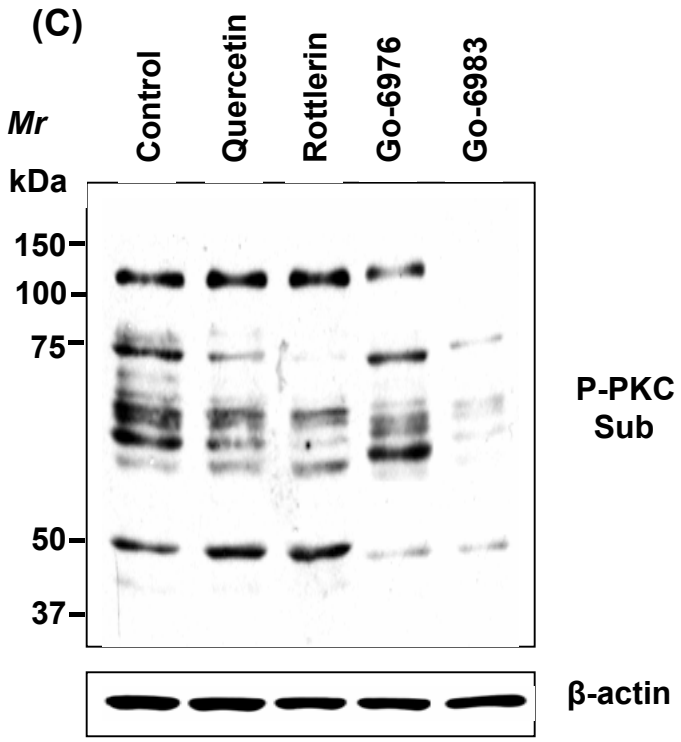
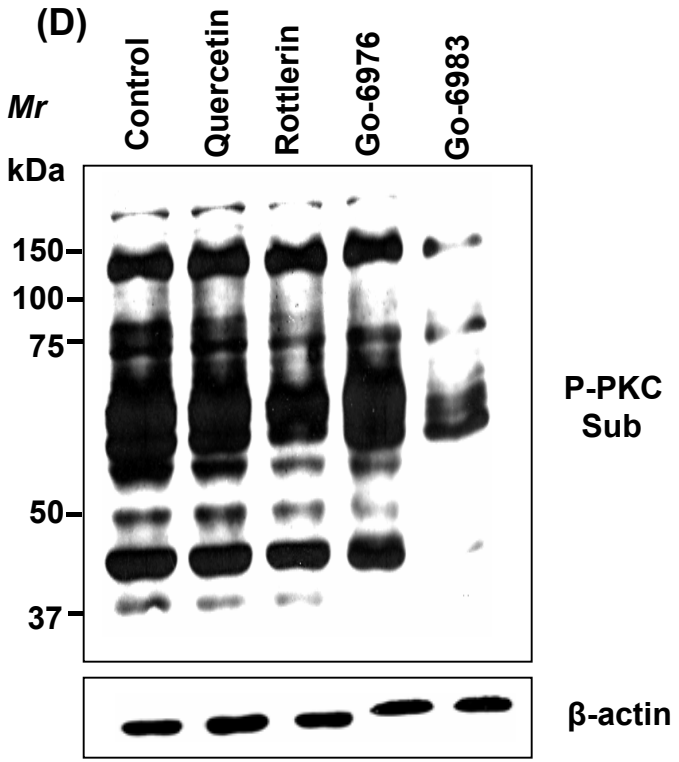
β-actin

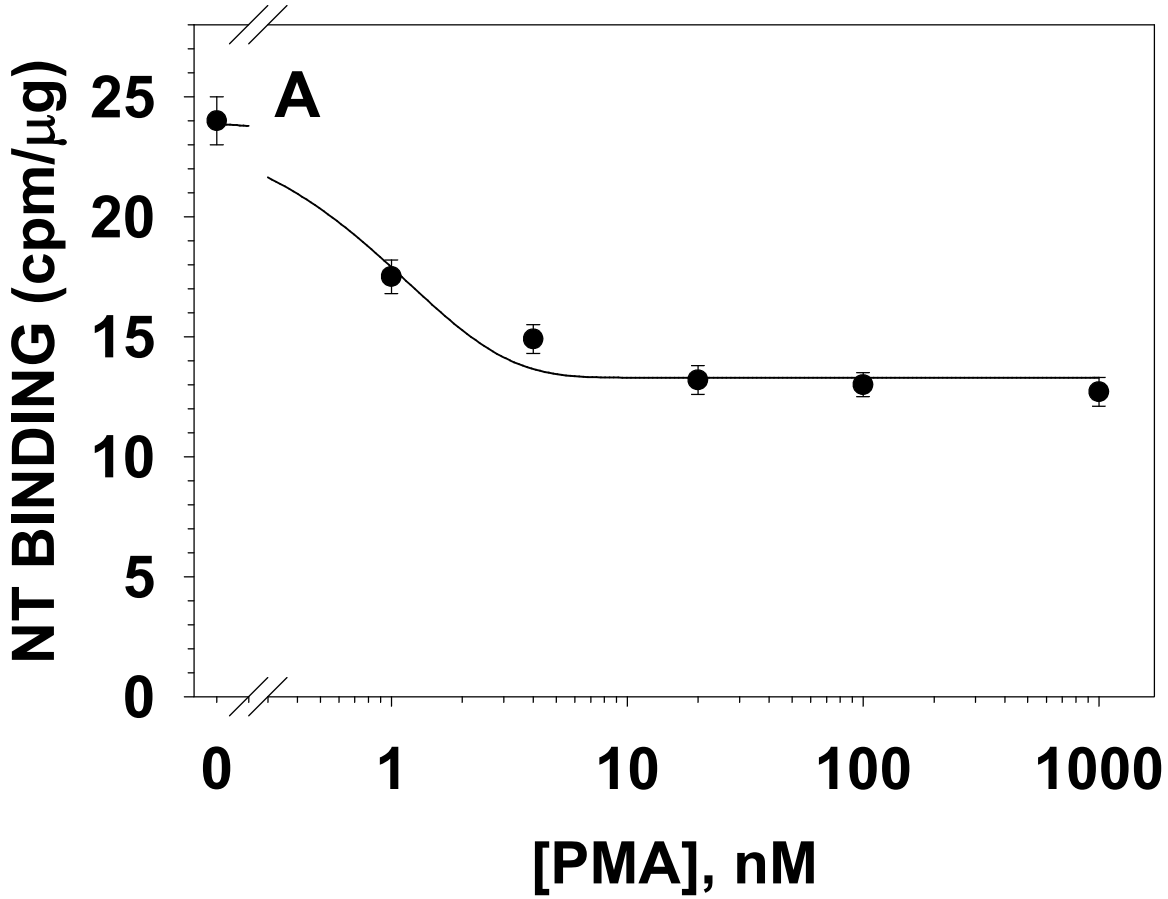
Figure


(C)
Control
Quercetin
Rottlerin
Go-6976
Go-6983
Mr
kDa
150
100
75
50
37
P-PKC
Sub
β-actin

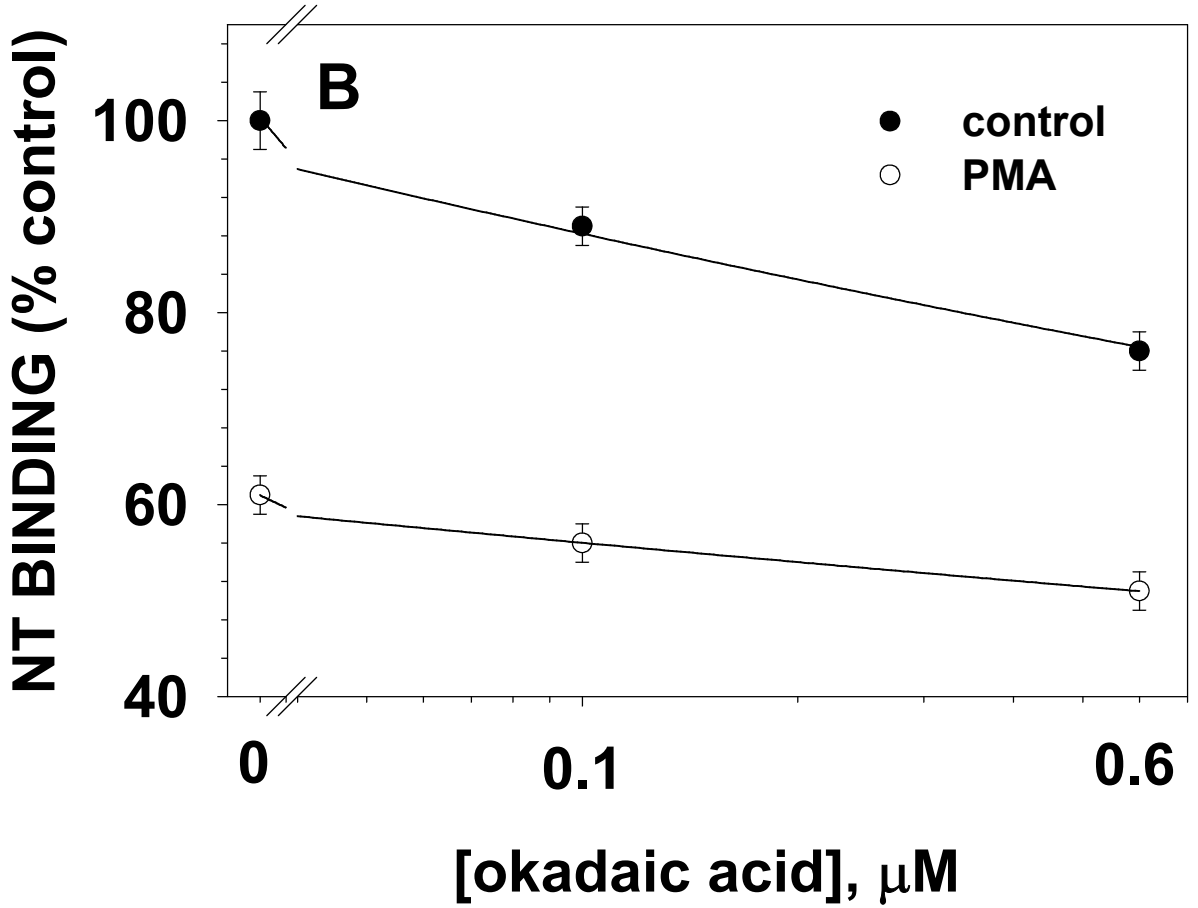
Figure


(D)
Control
Quercetin
Rottlerin
Go-6976
Go-6983
Mr
kDa
150
100
75
50
37
P-PKC
Sub
β-actin

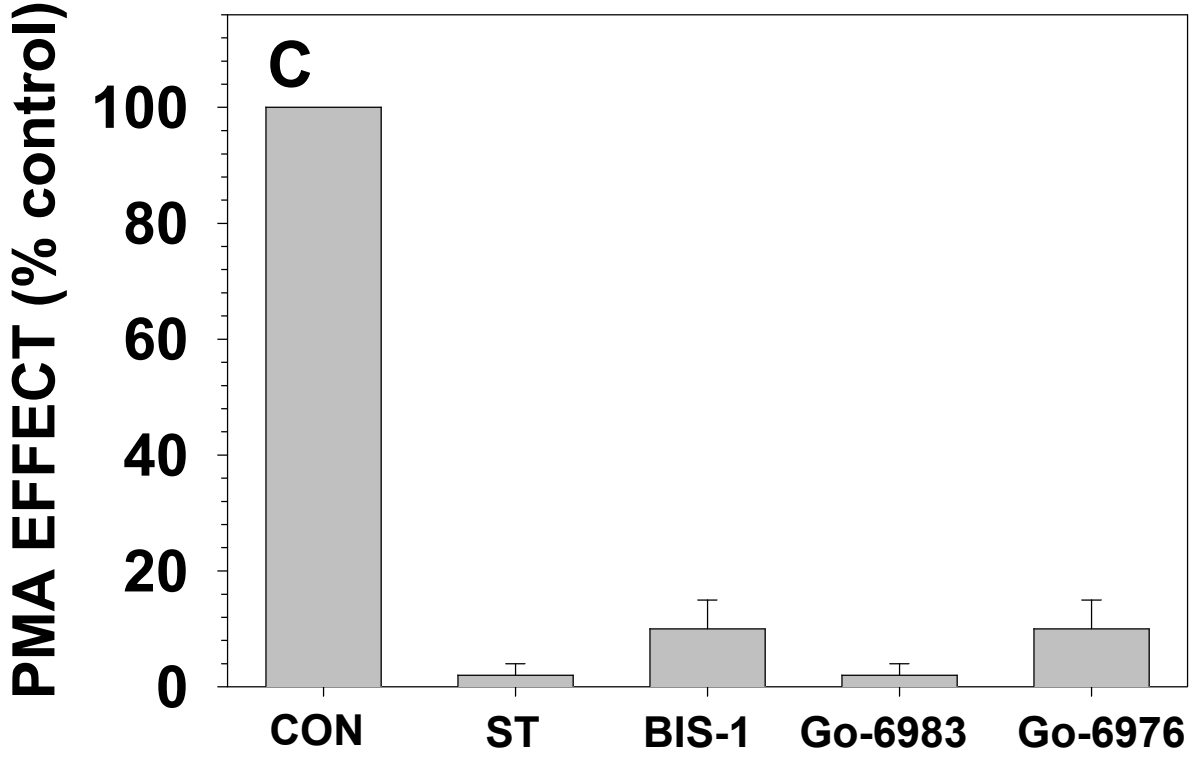
Figure



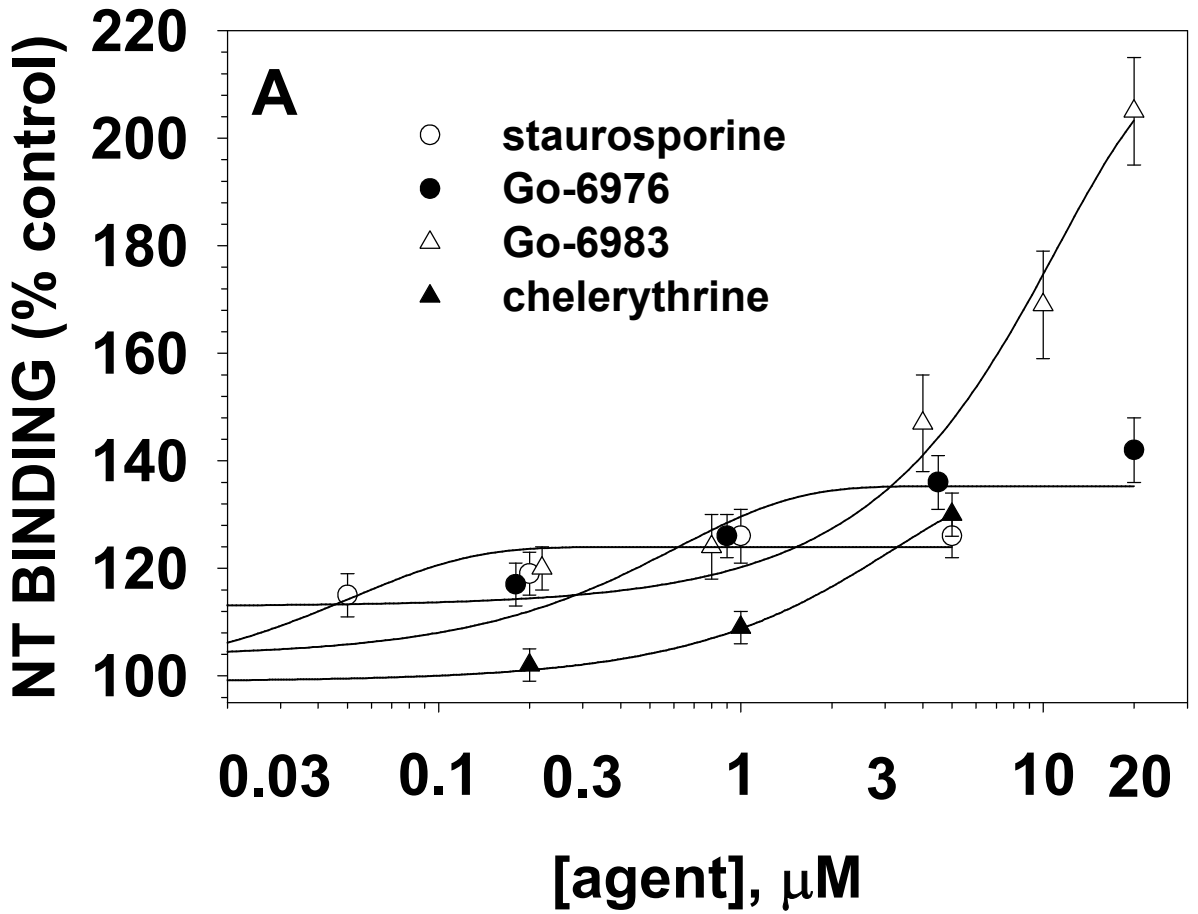
Figure


B
control
PMA
NT BINDING (% control)
100
80
60
40
0
0.1
0.6
[okadaic acid], μM

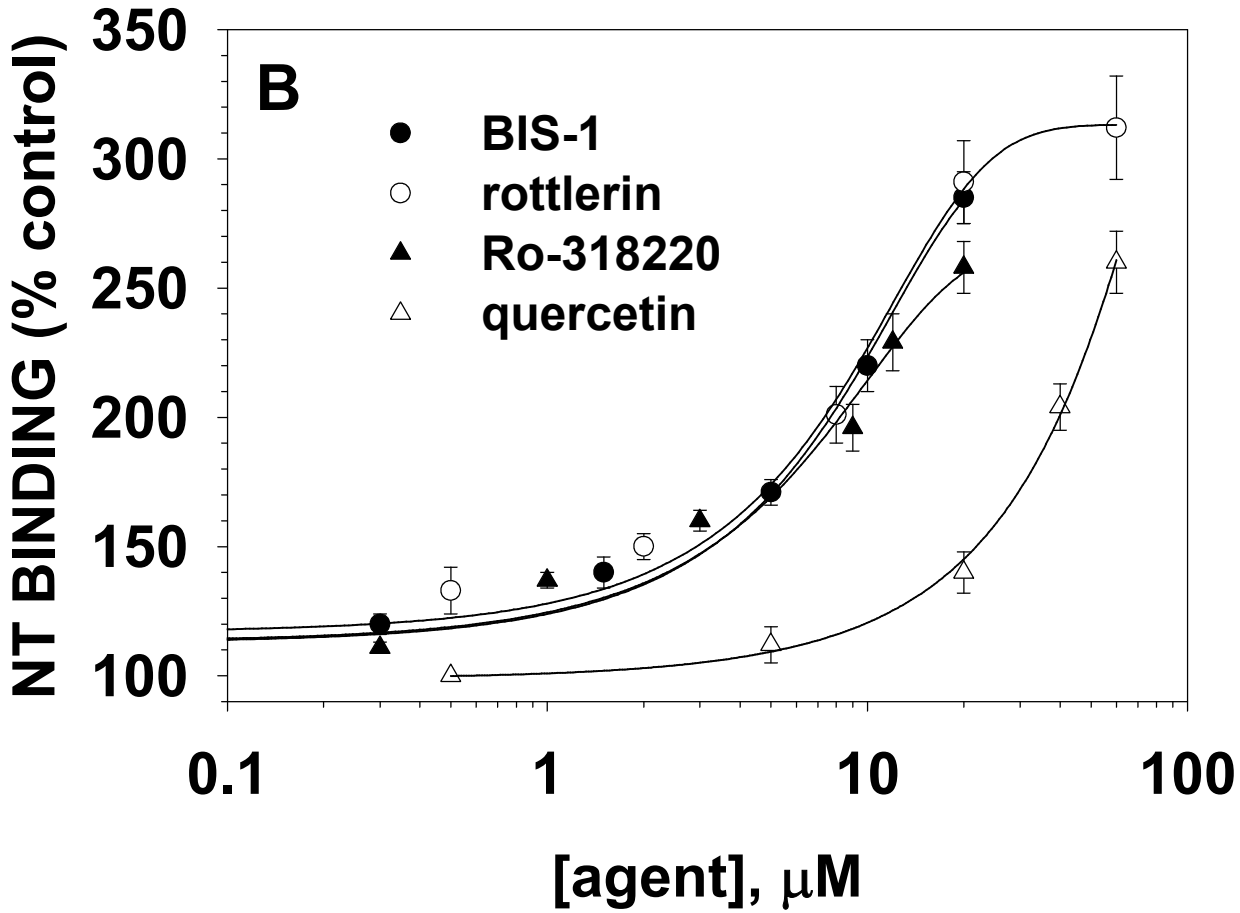
Figure


C
PMA EFFECT (% control)
100
80
60
40
20
0
CON
ST
BIS-1
Go-6983
Go-6976

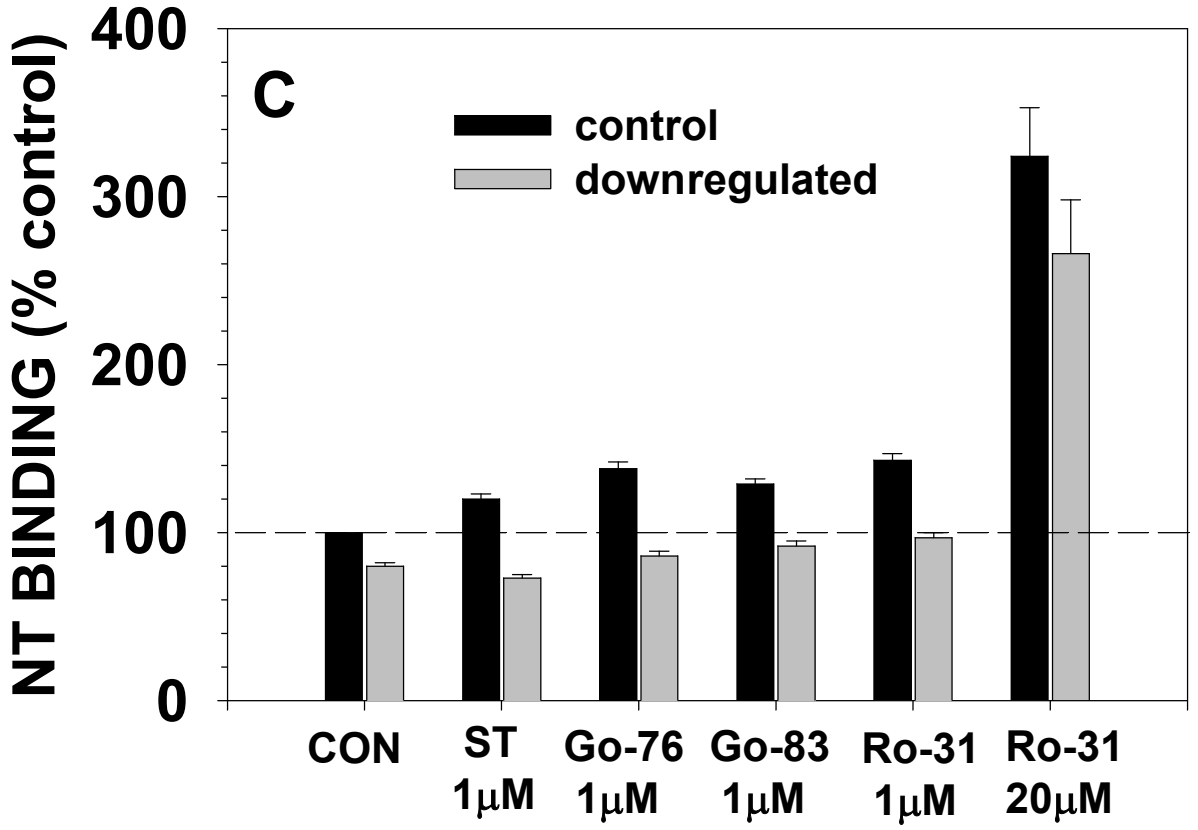
Figure


A
staurosporine
Go-6976
Go-6983
chelerythrine
NT BINDING (% control)
220
200
180
160
140
120
100
0.03
0.1
0.3
1
3
10
20
[agent], μM

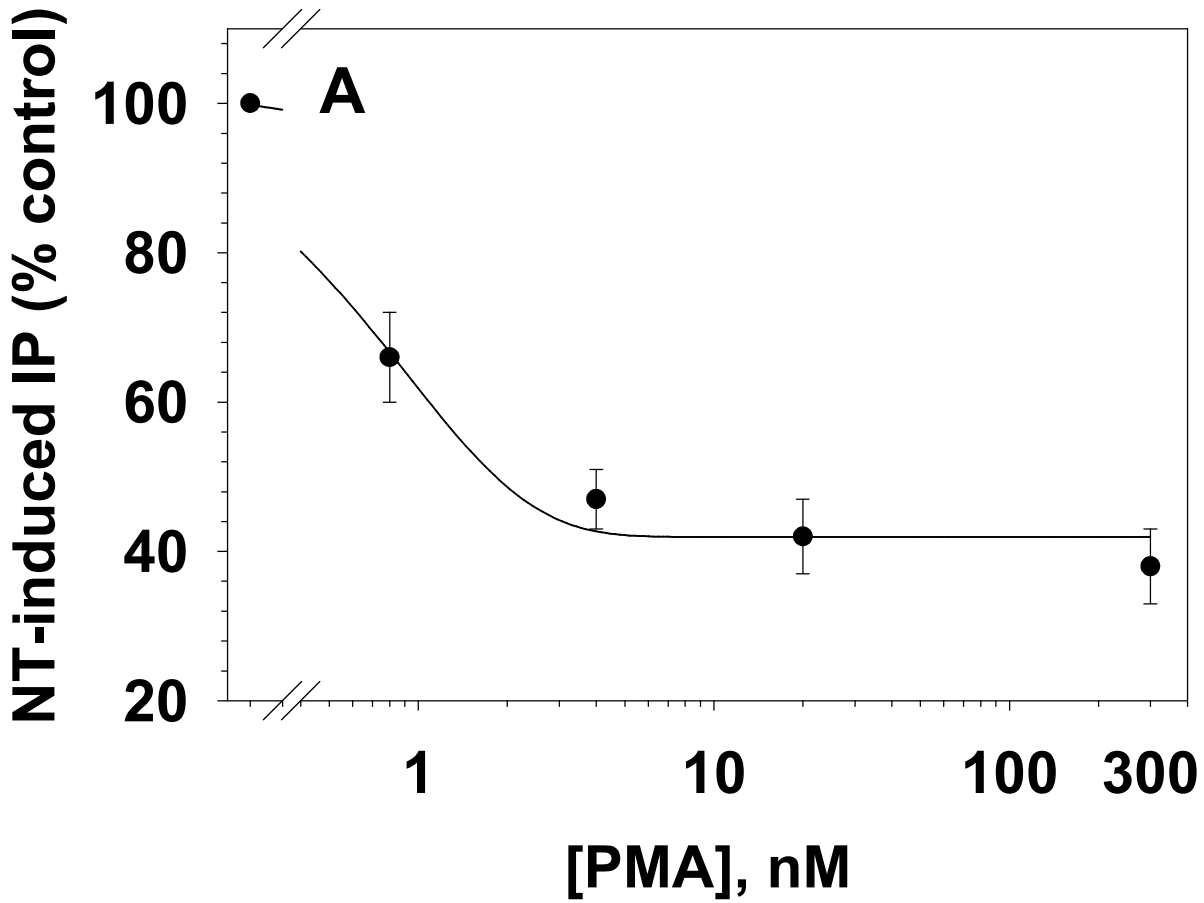
Figure


B
BIS-1
rottlerin
Ro-318220
quercetin
NT BINDING (% control)
350
300
250
200
150
100
0.1
1
10
100
[agent], μM

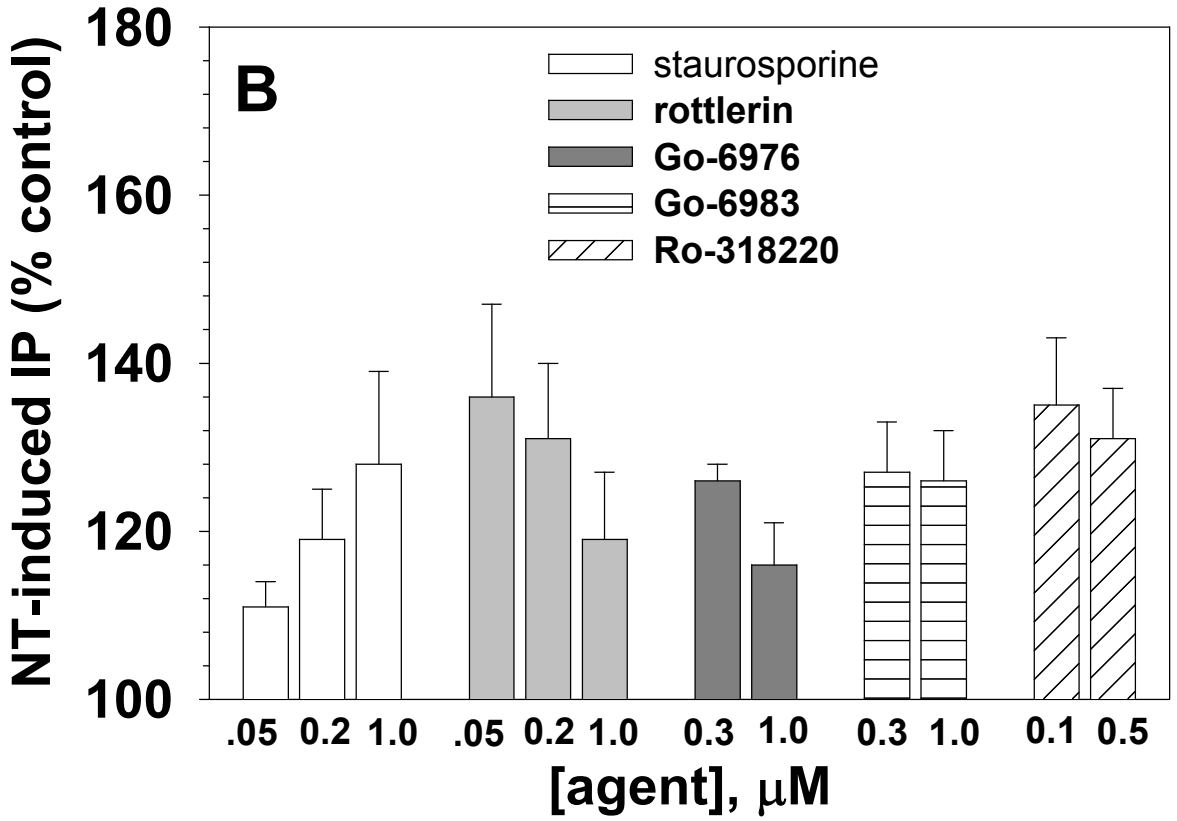
Figure


C
control
downregulated
NT BINDING (% control)
400
300
200
100
0
CON
ST 1μM
Go-76 1μM
Go-83 1μM
Ro-31 1μM
Ro-31 20μM

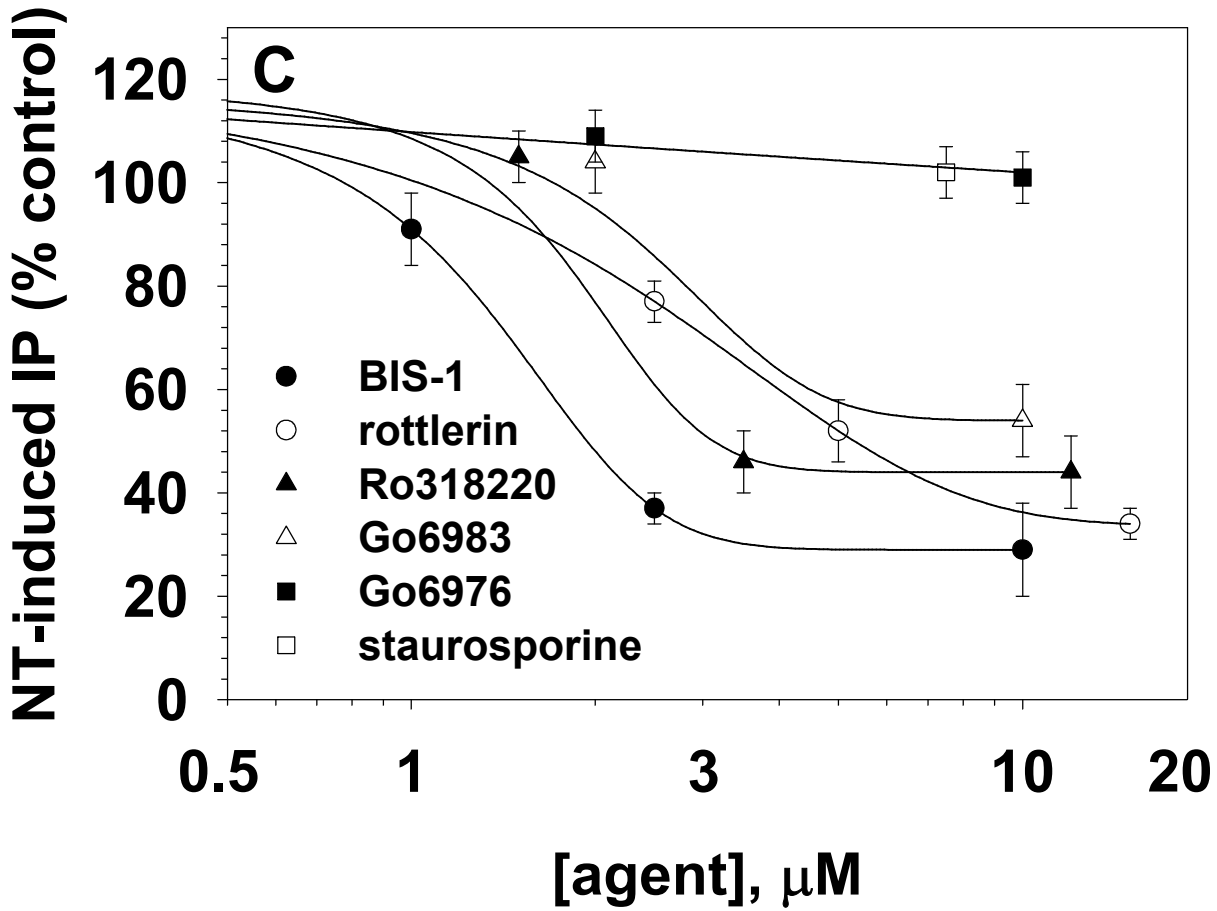
Figure



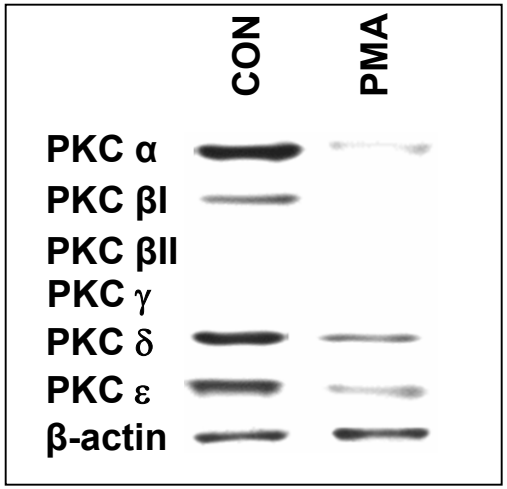
Figure


B
staurosporine
rottlerin
Go-6976
Go-6983
Ro-318220
NT-induced IP (% control)
180
160
140
120
100
.05 0.2 1.0 .05 0.2 1.0 0.3 1.0 0.3 1.0 0.1 0.5
[agent], μM

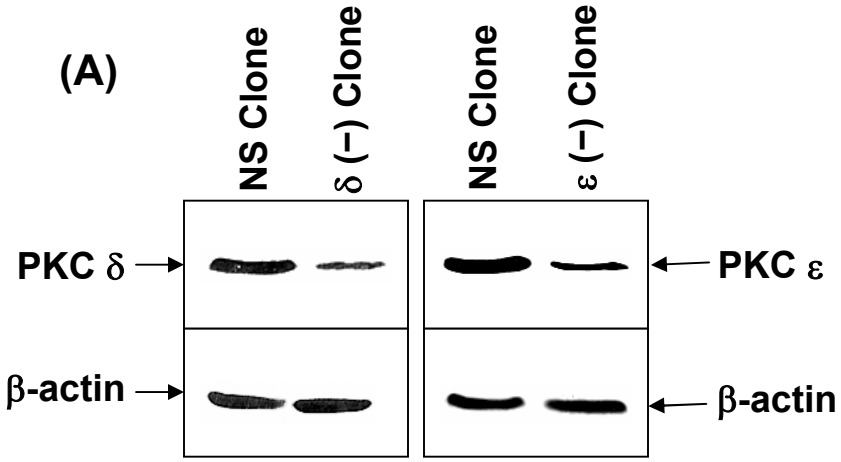
Figure


C
NT-induced IP (% control)
120
100
80
60
40
20
0
0.5
1
3
10
20
[agent], μM
BIS-1
rottlerin
Ro318220
Go6983
Go6976
staurosporine

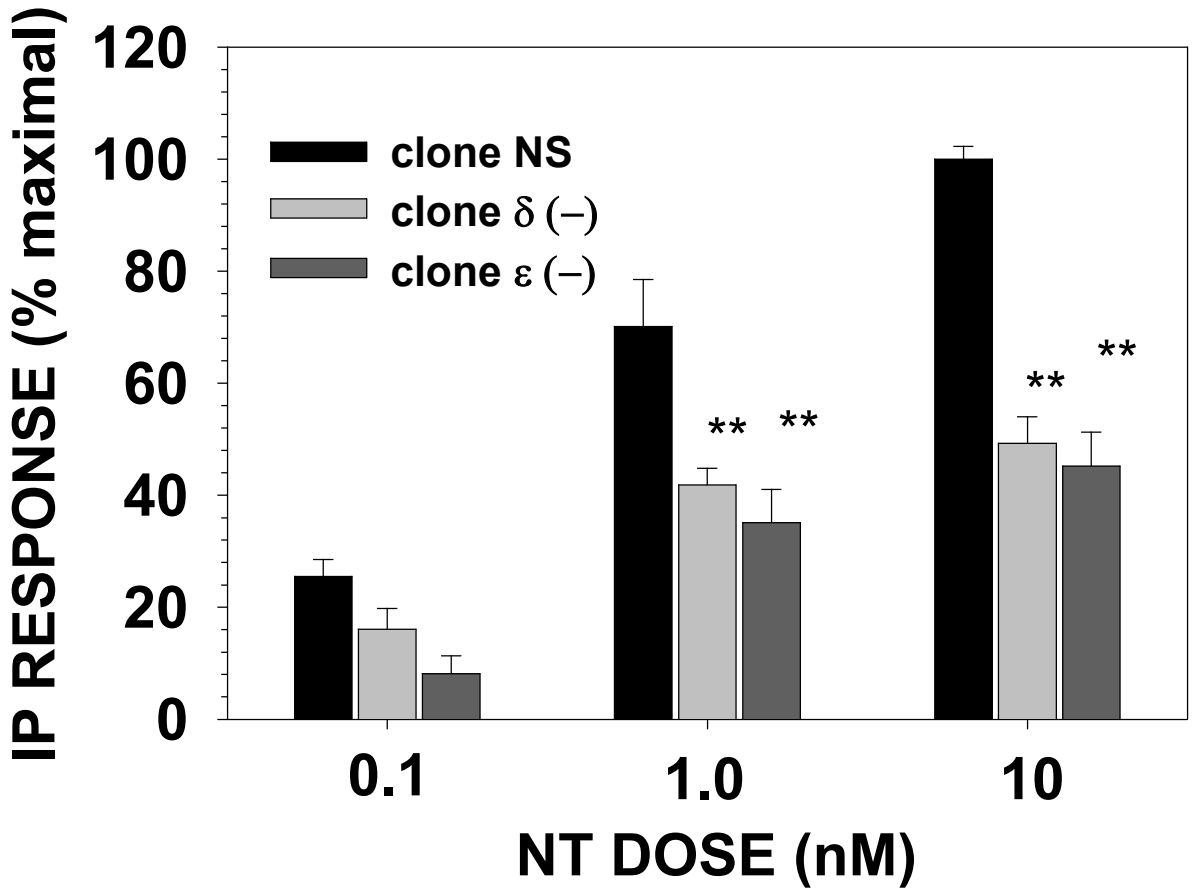
Figure



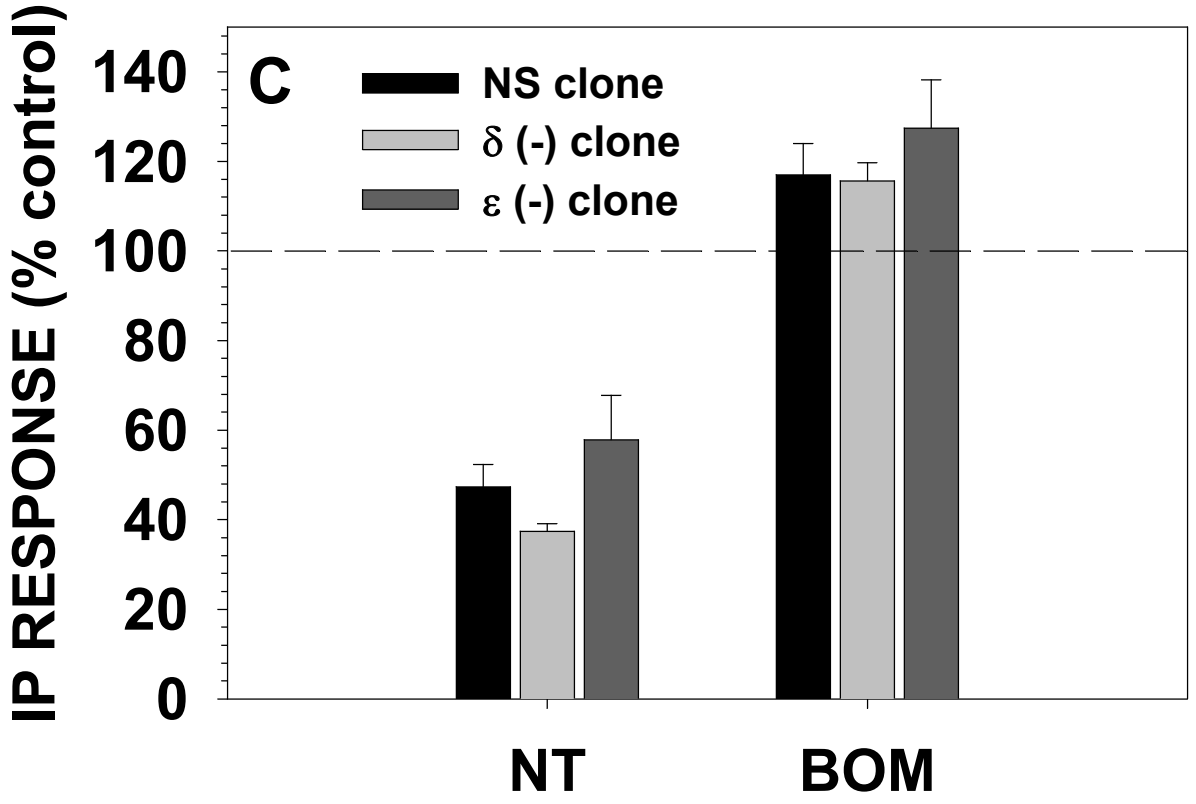
Figure



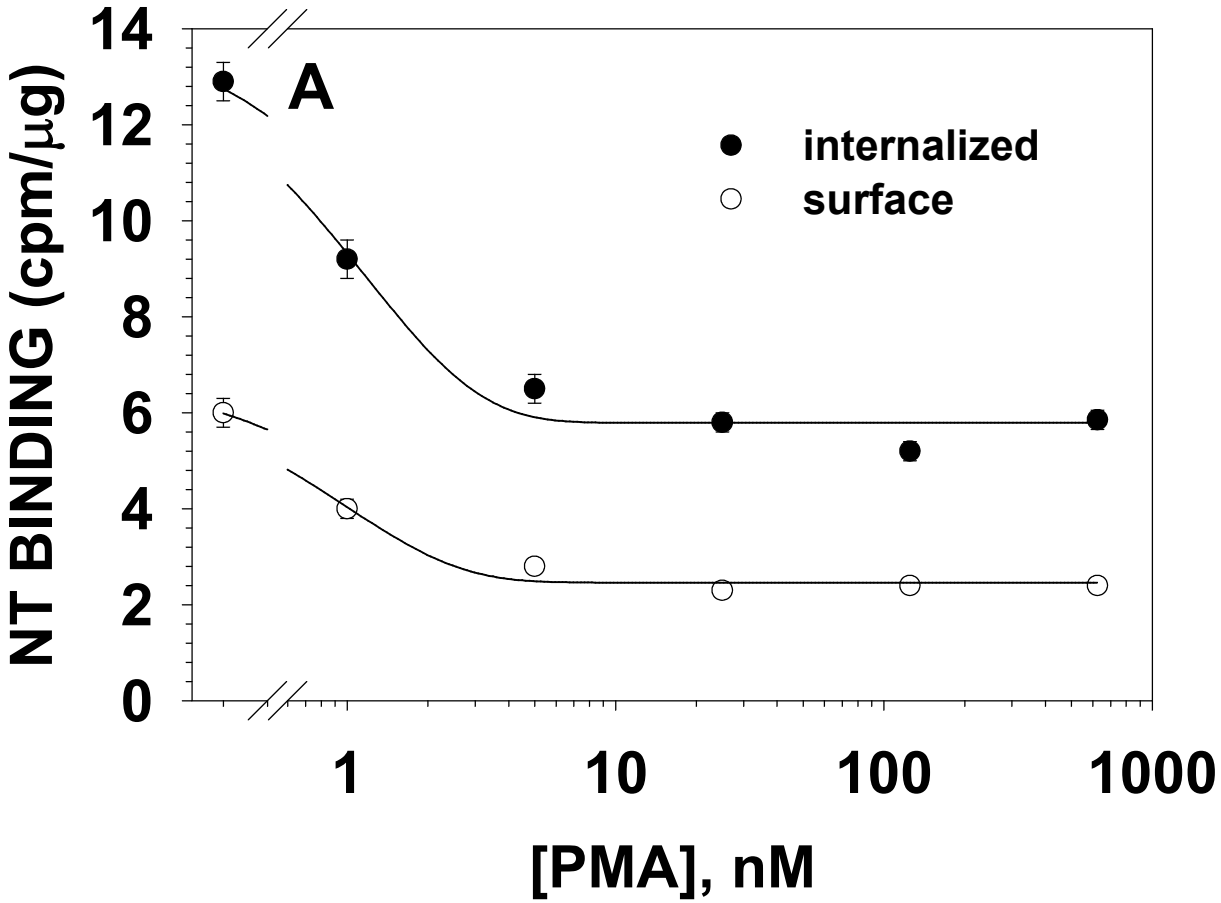
Figure


IP RESPONSE (% maximal)
120
100
80
60
40
20
0
clone NS
clone δ (–)
clone ε (–)
**
**
**
**
0.1
1.0
10
NT DOSE (nM)

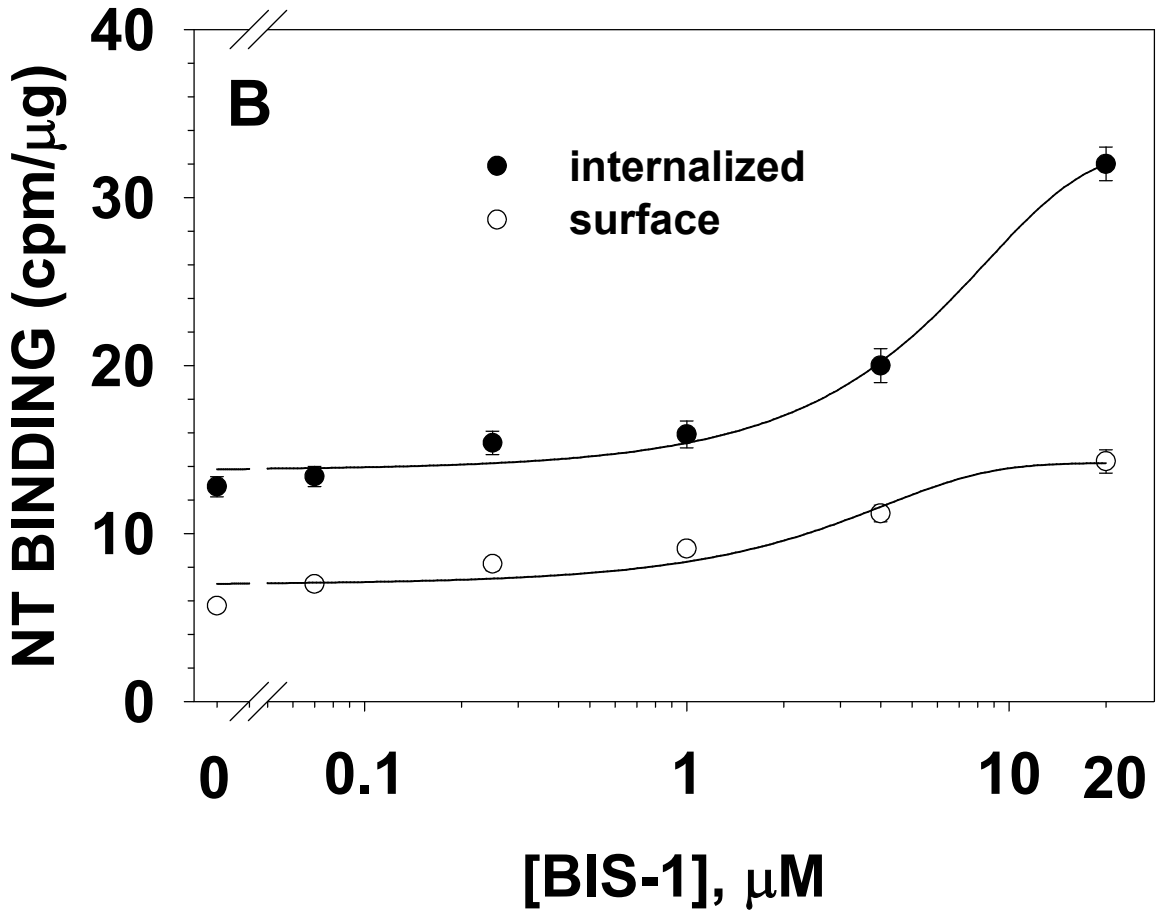
Figure


C
NS clone
δ (-) clone
ε (-) clone
IP RESPONSE (% control)
140
120
100
80
60
40
20
0
NT
BOM

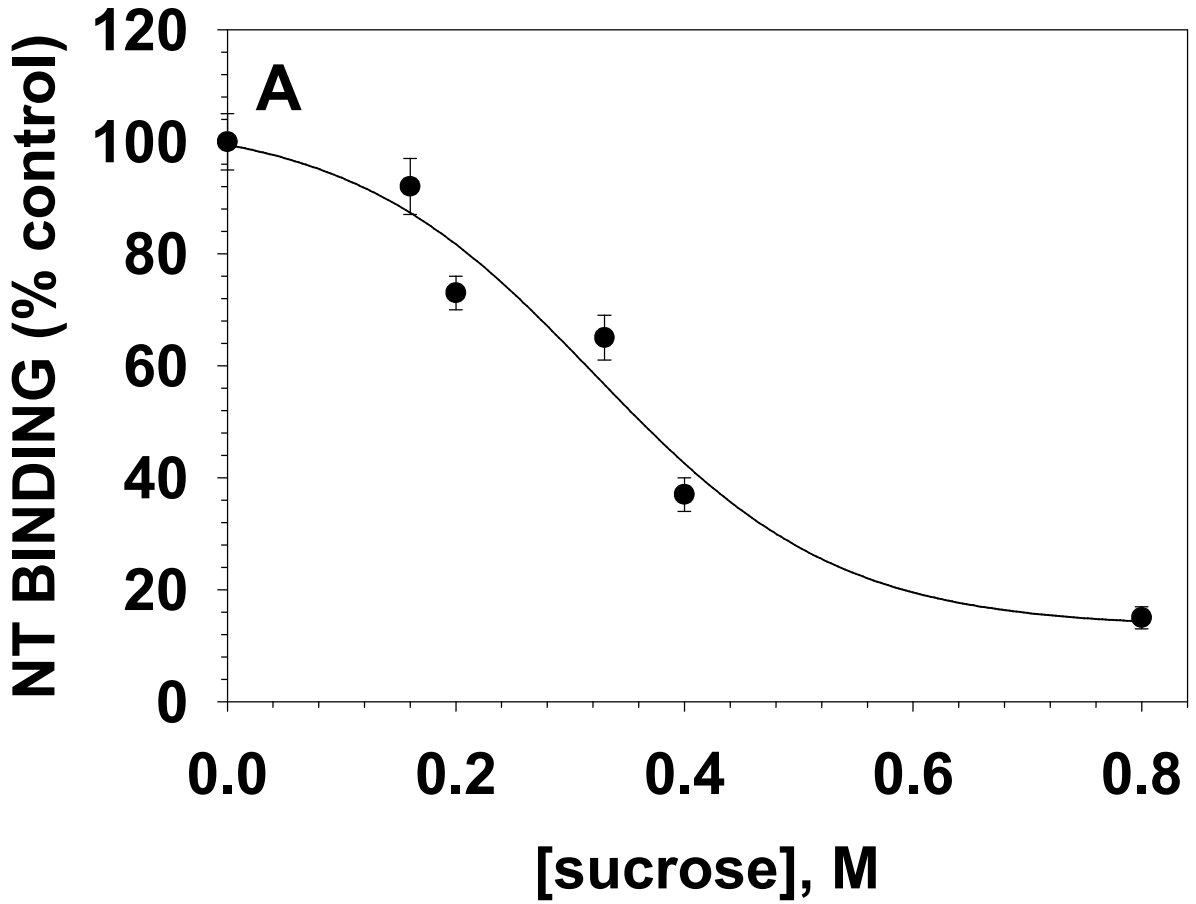
Figure


A
internalized
surface
NT BINDING (cpm/μg)
14
12
10
8
6
4
2
0
1
10
100
1000
[PMA], nM

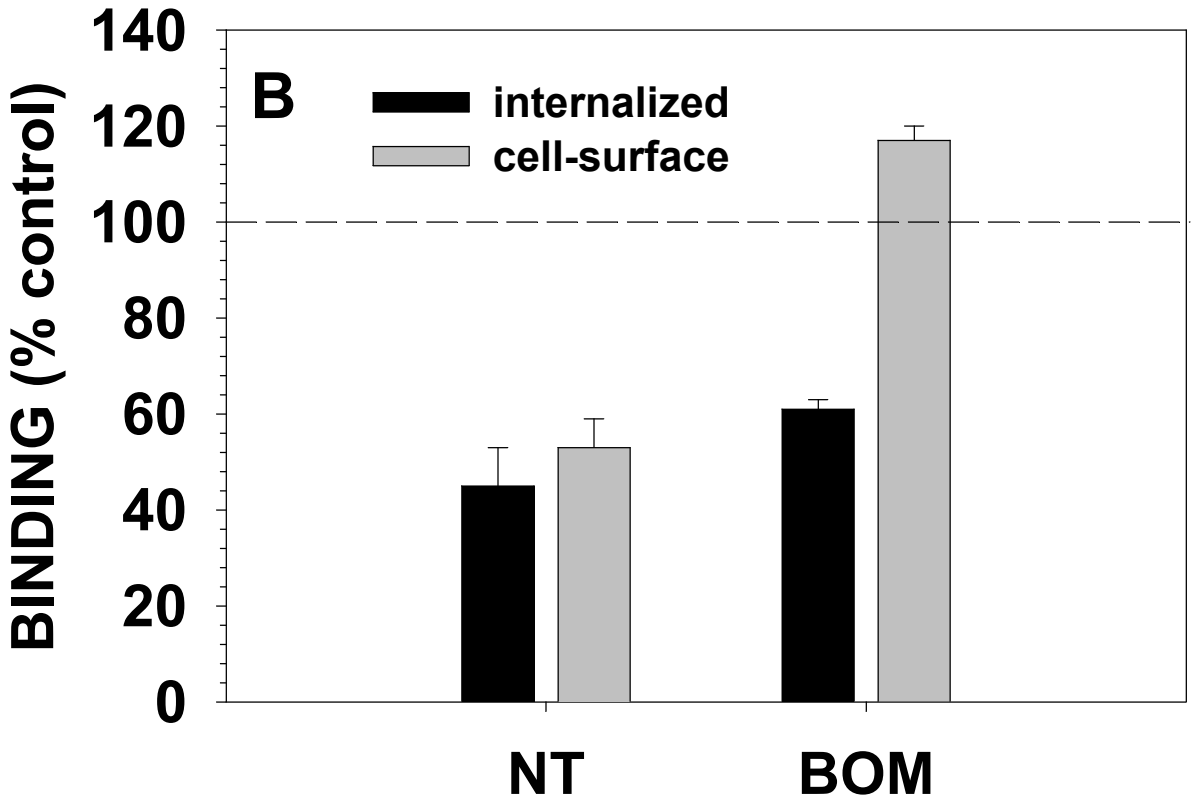
Figure


B
internalized
surface
NT BINDING (cpm/μg)
40
30
20
10
0
0
0.1
1
10
20
[BIS-1], μM

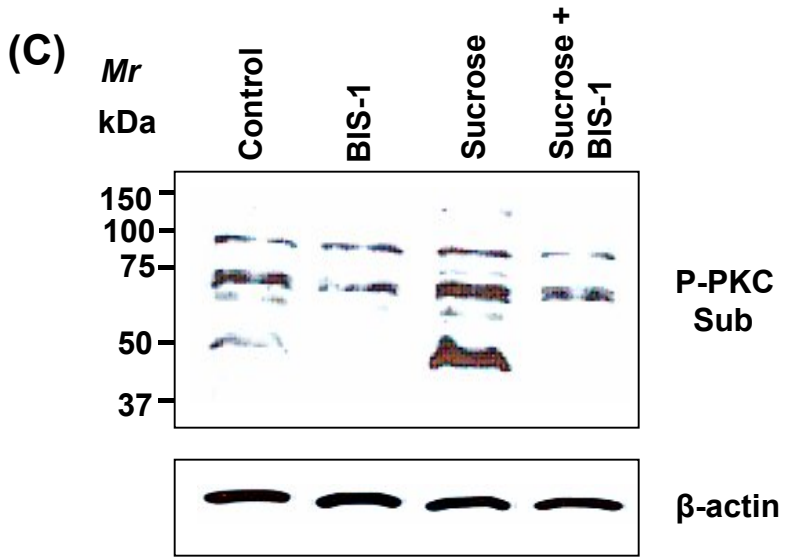
Figure


A
NT BINDING (% control)
120
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
[sucrose], M

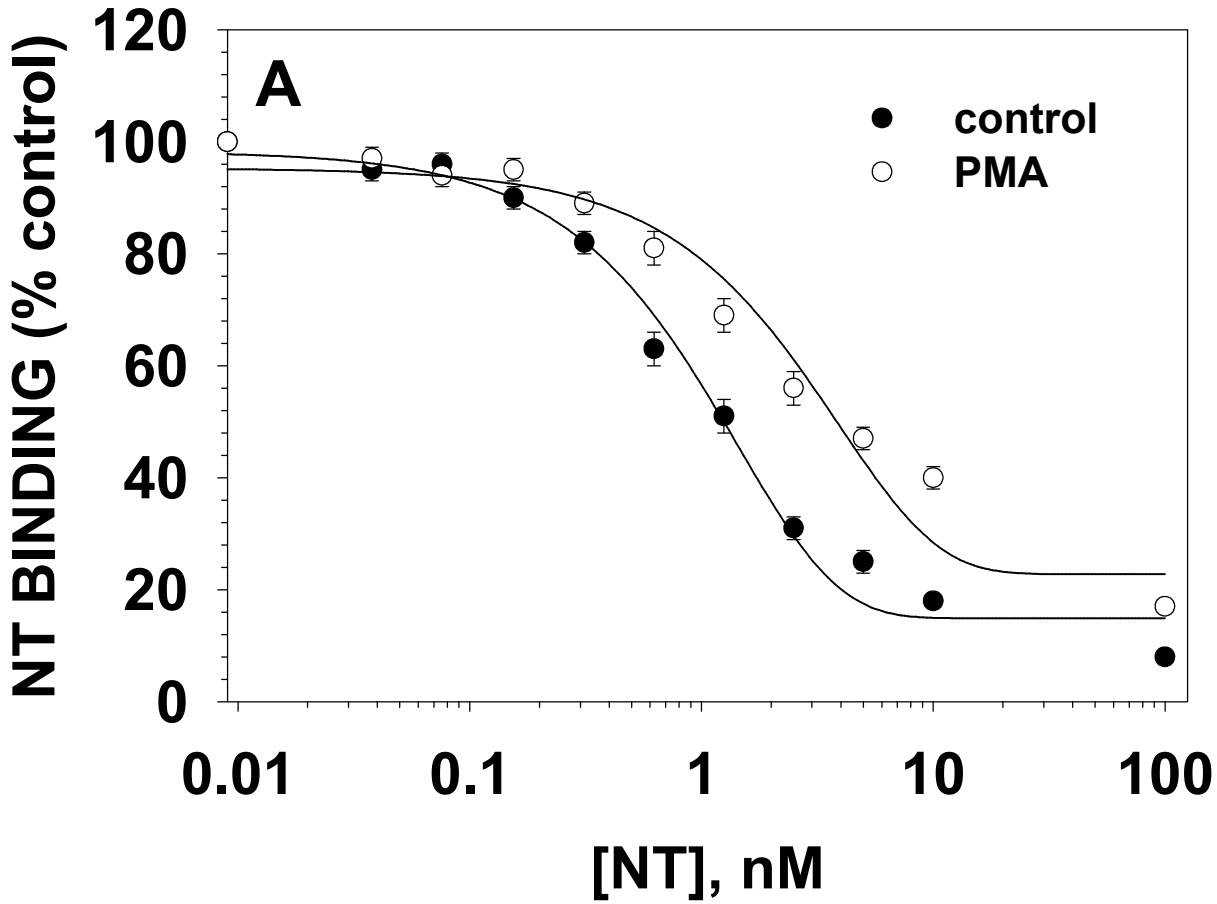
Figure


B
internalized
cell-surface
BINDING (% control)
140
120
100
80
60
40
20
0
NT
BOM

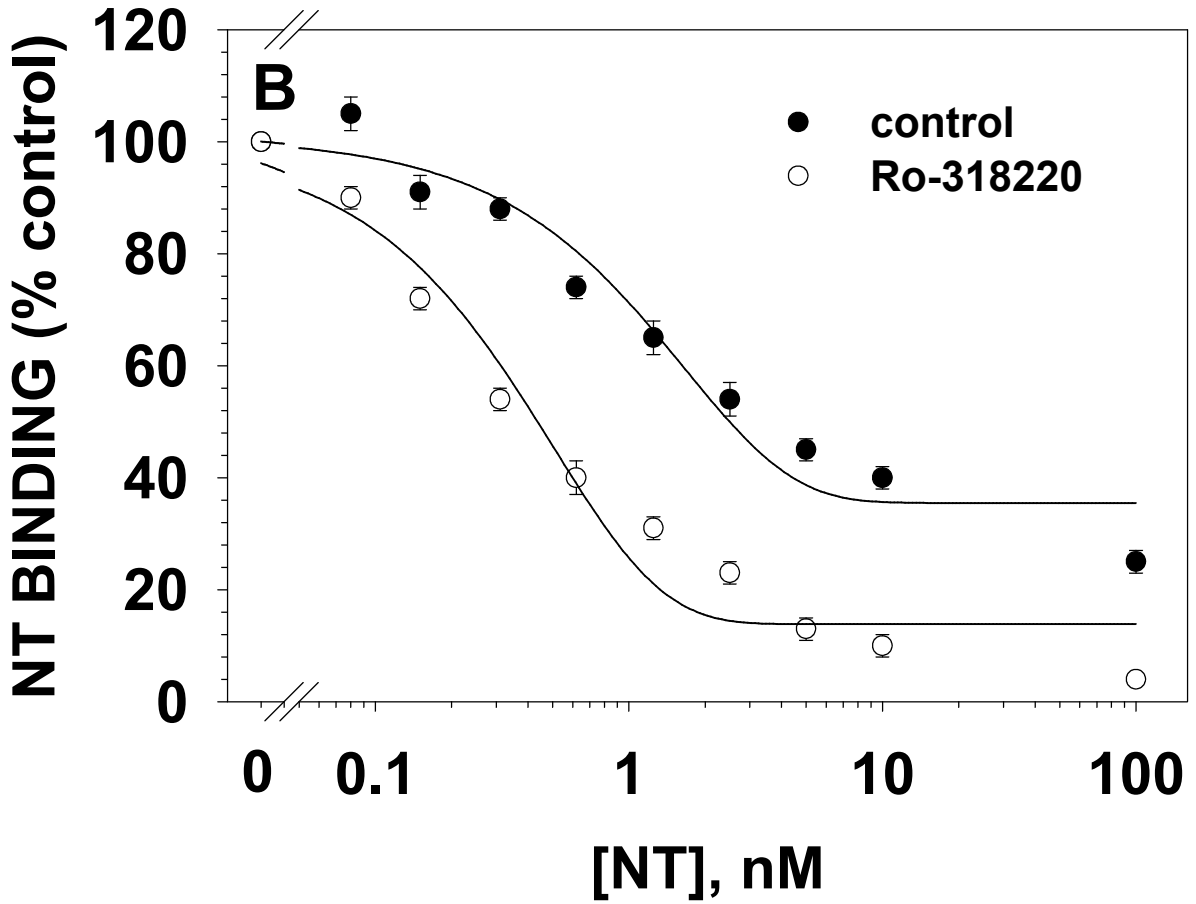
Figure


(C)
Mr
kDa
Control
BIS-1
Sucrose
Sucrose + BIS-1
150
100
75
50
37
P-PKC Sub
β-actin

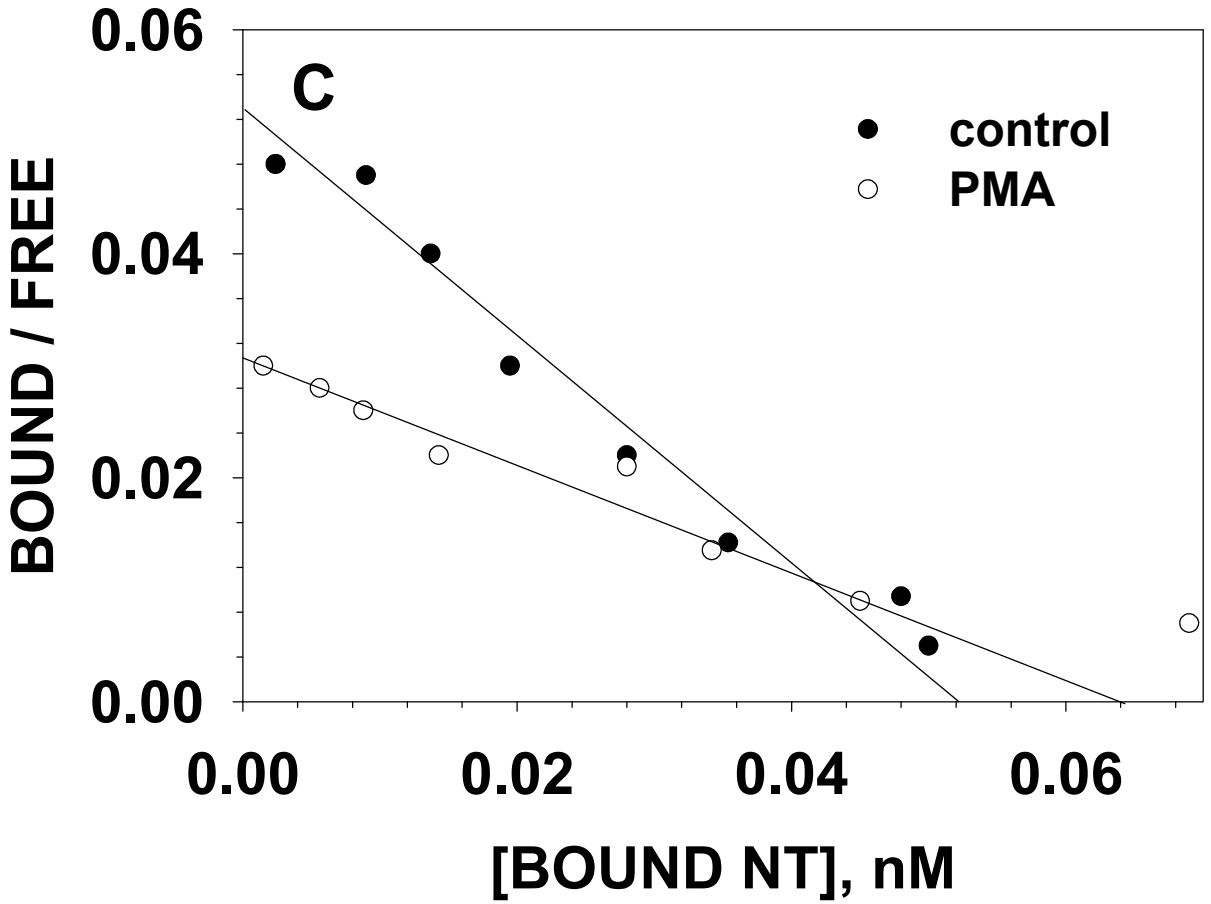
Figure


A
control
PMA
NT BINDING (% control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
[NT], nM

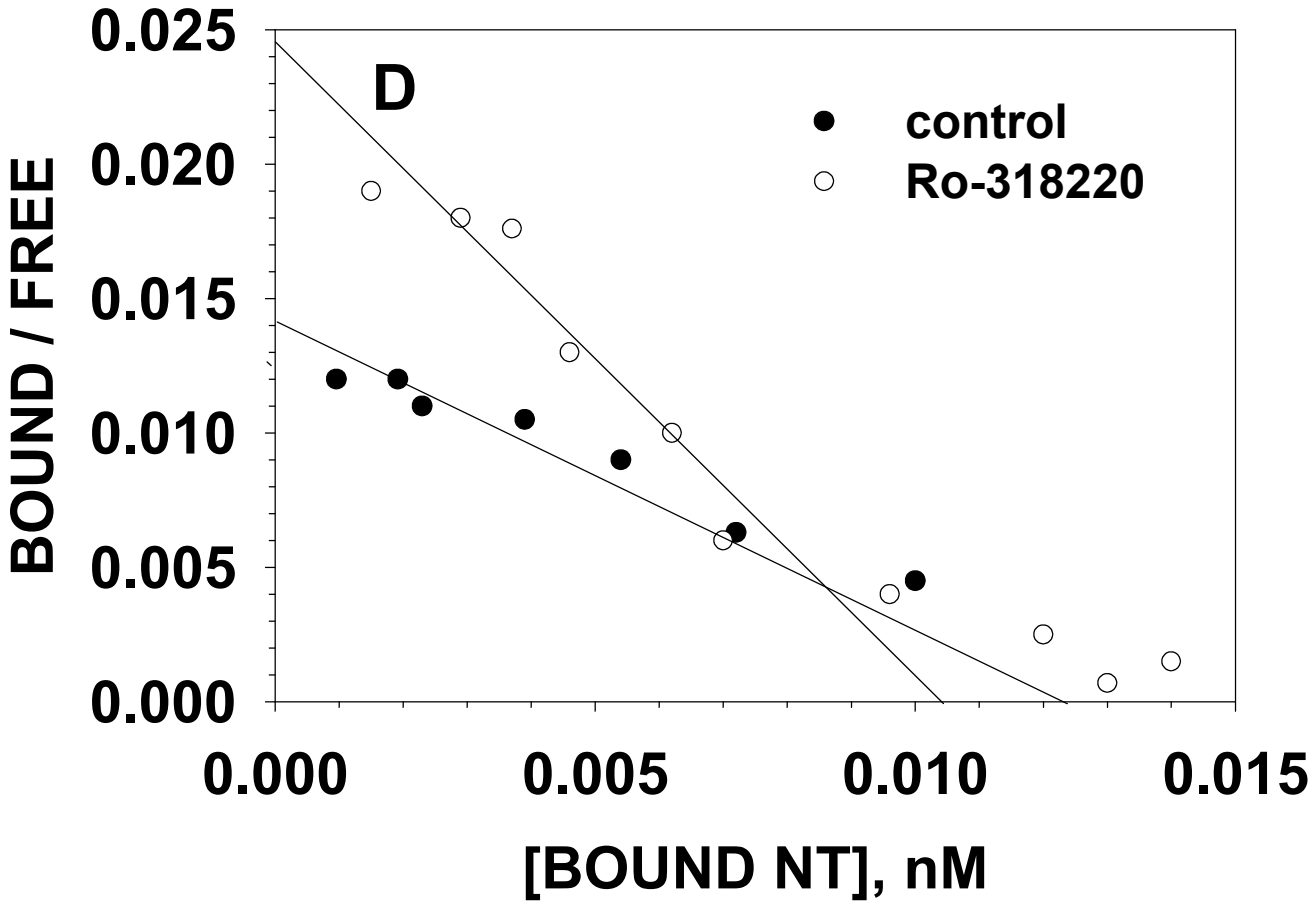
Figure


B
control
Ro-318220
NT BINDING (% control)
120
100
80
60
40
20
0
0
0.1
1
10
100
[NT], nM

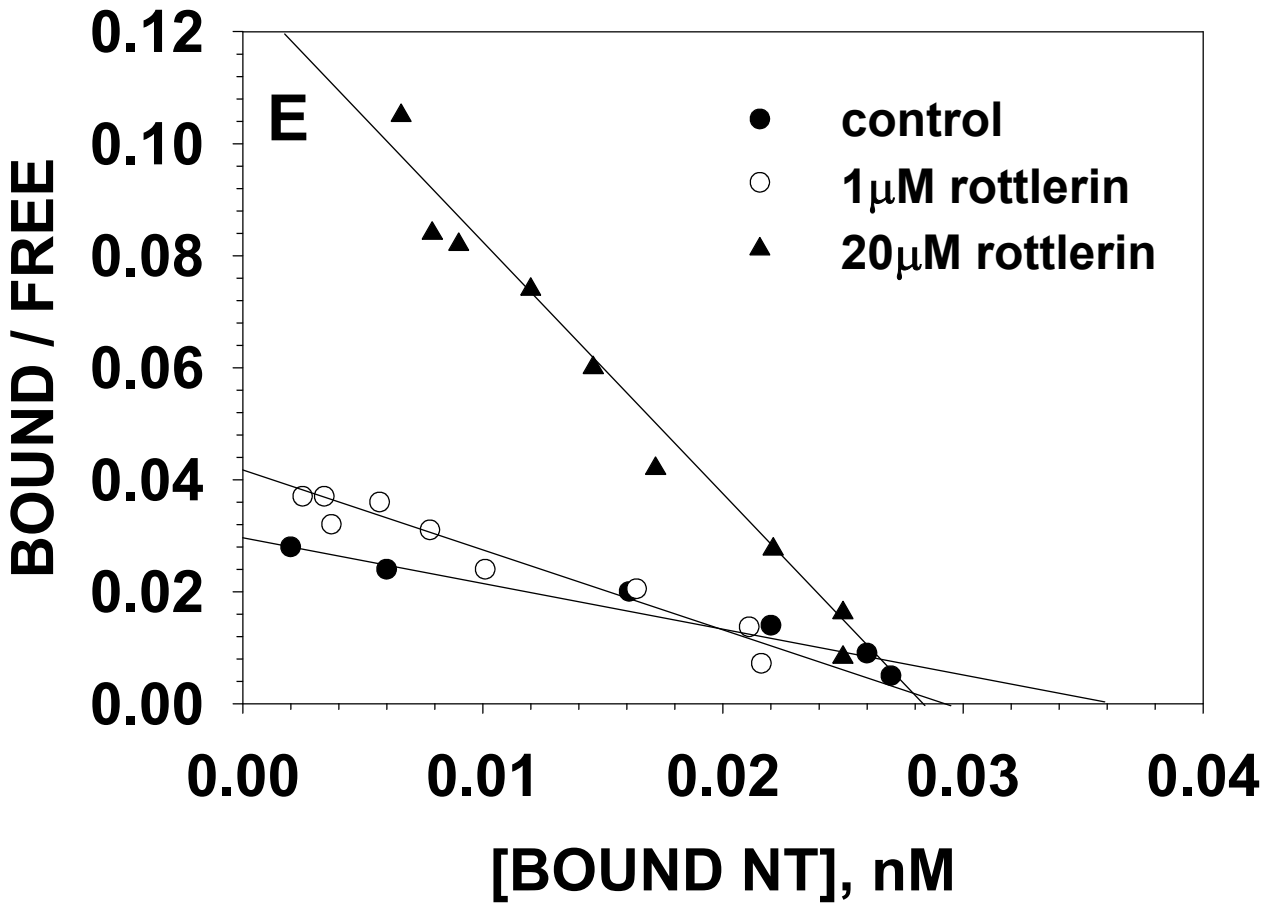
Figure


C
control
PMA
BOUND / FREE
0.06
0.04
0.02
0.00
0.00
0.02
0.04
0.06
[BOUND NT], nM

Figure


D
control
Ro-318220
BOUND / FREE
0.025
0.020
0.015
0.010
0.005
0.000
0.000
0.005
0.010
0.015
[BOUND NT], nM

Figure


E
control
1μM rottlerin
20μM rottlerin
BOUND / FREE
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00
0.01
0.02
0.03
0.04
[BOUND NT], nM